medical
library
9-25-67

The Journal of Laboratory and Clinical Medicine

VOL. 25 APRIL, 1940 No. 7

CLINICAL AND EXPERIMENTAL

DISTRIBUTION OF FREE AND CONJUGATED SULFANILAMIDE AND SULFAPYRIDINE BETWEEN CORPUSCLES AND PLASMA IN BOTH HUMAN AND RABBIT BLOOD*

LORENZ HANSEN, PH.D., PHILADELPHIA, PA.

IN A STUDY of the therapeutic use of sulfanilamide for gonococcic infections in men[1] the observation was made that the concentrations of both the free and the conjugated drug were almost invariably higher in the corpuscles than in the plasma. This behavior appears to be out of harmony with the idea that sulfanilamide is distributed in proportion to the water content of the tissues.[2] I pointed out that this behavior suggests the possibility of a loose union between the drug and something within the corpuscles or in its membrane.

Furthermore, there appeared to be a correlation between the cure for gonococcic infection and the ratios of the amount of the conjugated form of the drug in the corpuscles to the amount in the plasma. The low ratios were associated with positive cures and the high ratios with failures. For these reasons, and because of the limited number of the individual observations made, and because these observations were all made in connection with only one type of infection, the study on distribution has been extended to individuals treated with sulfanilamide for different infections, including a number of individuals treated repeatedly over extended periods. In addition, the study was extended to include the distribution of sulfapyridine in man infected with different organisms, of sulfanilamide and sulfapyridine in normal rabbits, and of these two drugs when added to human blood in vitro.

Methods.—Data presented in Tables I and II were obtained from analyses which were made according to the method of Marshall.[3] All other data were

*From the Department of Physiological Chemistry of Jefferson Medical College, Philadelphia, Pa.

Received for publication, June 29, 1939.

TABLE I

DISTRIBUTION OF FREE AND CONJUGATED SULFANILAMIDE BETWEEN CORPUSCLES AND PLASMA IN BLOOD OF PATIENTS DURING TREATMENT FOR VARIOUS INFECTIONS

SEX	INFECTION	IN WHOLE BLOOD			IN PLASMA			PLASMA	IN PLASMA			CORPUSCLES	IN CORPUSCLES			RATIO OF AMOUNT IN		
		FREE SULF.*	CONJUGATED SULF.	TOTAL SULF.	FREE SULF.	CONJUGATED SULF.	TOTAL SULF.	VOLUMES	FREE SULF.	CONJUGATED SULF.	TOTAL SULF.	VOLUMES	FREE SULF.	CONJUGATED SULF.	TOTAL SULF.	100 ML. OF CORPUSCLES		
																100 ML. OF PLASMA		
		mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	Free Sulf.	Conjugated Sulf.	Total Sulf.
M	β hemolytic streptococci	6.72	1.83	8.55	5.71	1.34	7.05	70.2	59.6	51.4	59.0	29.8	40.4	48.6	41.0	1.59	2.22	1.67
M	β hemolytic streptococci	2.46	1.52	3.98	1.67	0.59	2.26	64.6	43.1	25.0	36.7	35.4	56.9	75.0	63.4	2.38	5.45	3.14
M	β hemolytic streptococci	3.23	1.69	4.92	2.42	1.58	4.0	61.5	46.1	57.4	50.0	38.5	53.9	42.6	50.0	2.59	1.64	2.08
F	β hemolytic streptococci	3.39	2.55	5.94	2.82	1.30	4.12	63.4	52.8	32.5	43.9	36.6	47.2	67.5	56.1	1.55	3.62	2.01
F	β hemolytic streptococci	3.79	1.52	5.31	3.31	1.54	4.85	76.1	66.5	58.0	69.5	23.9	33.5	42.0	30.5	1.60	1.74	1.40
F	β hemolytic streptococci	1.94	0.64	2.58	1.67	0.11	1.78	73.3	62.9	12.5	50.4	26.7	37.1	87.5	49.6	1.62	18.9	2.68
F	B. coli	6.71	1.01	7.72	4.42	0.58	5.0	66.7	43.8	38.6	43.1	33.3	56.2	61.4	56.9	2.56	3.20	2.63
F	B. coli	7.80	2.51	10.31	7.13	1.47	8.6	58.0	53.0	33.8	48.4	42.0	47.0	66.2	51.6	1.23	2.69	1.48
M	Streptococcus viridans	4.34	1.96	6.3	3.72	1.21	4.93	72.7	62.4	44.9	57.0	27.3	37.6	55.1	43.0	1.61	3.26	2.01
M	Streptococcus viridans	10.05	1.25	11.3	9.20	0.35	9.55	62.4	57.1	17.6	52.7	37.6	42.9	82.4	47.3	1.25	7.82	1.49
F	Gonorrheal arthritis	0.89	0.75	1.64	0.53	0.12	0.65	67.8	40.5	10.7	26.8	32.2	59.5	89.3	73.1	1.47	8.37	2.73
M	Undulant fever	3.65	0.65	4.3	2.08	0.42	2.50	65.25	37.2	41.5	37.9	34.75	62.8	58.5	62.1	3.17	2.62	3.08

*Sulf.=Sulfanilamide.

prepared from analyses according to the modified procedure of the above method by Marshall and Litchfield.[4]

Experimental.—Distribution of Sulfanilamide in Human Blood In Vivo.—Table I shows typical examples of the distribution of sulfanilamide between corpuscles and plasma in a series of individuals, giving sex, type of infection, blood and plasma levels, volumes per cent of plasma, and percentage of the drug in the plasma, volumes per cent of the corpuscles and percentage of the drug in the corpuscles, and the ratio of the concentration of the drug in the corpuscles to that in the plasma. It may be seen that variation in the distribution of the drug between corpuscles and plasma cannot be ascribed to either sex or type of infection. By contrasting the per cent of drug in the plasma and corpuscles with the corresponding volumes per cent, one may readily see that in all the cases cited the per cent of the drug in the corpuscles is noticeably higher than the volumes per cent of the corpuscles, and that the per cent of the drug in the plasma is distinctly lower than the volumes per cent of the plasma, which indicates a decidedly unequal distribution of the drug between corpuscles and plasma, and a consistently higher concentration in the corpuscles. The quantitative difference in the distribution is indicated by the ratios of the concentration in the corpuscles to that in the plasma, ranging from 1.23 to 3.17 for free sulfanilamide, from 1.64 to 18.9 for conjugated drug, and from 1.48 to 3.14 for total drug. It should be noted that the ratio for conjugated drug is generally high when the level of the conjugated drug in the plasma is low, especially when the level in the whole blood is relatively high.

TABLE II

VARIATION IN DISTRIBUTION OF FREE AND CONJUGATED SULFANILAMIDE BETWEEN CORPUSCLES AND PLASMA OF BLOOD OF PATIENTS DURING TREATMENT

Case 1. Male—Beta hemolytic streptococcus infection

DAYS TREATED		2	3	5	6	8	9	10
Ratio of amount in	Free sulfanilamide	1.98	1.80	1.74	0.86	1.59	1.90	1.92
	Conjugated sulfanilamide	0.50	0.71	1.14	4.25	2.22	1.30	2.02
100 ml. of corpuscles								
100 ml. of plasma	Total sulfanilamide	1.58	1.56	1.61	1.57	1.67	1.79	1.95

Case 2. Female—B. coli infection

DAYS TREATED		2	4	5	6	9
Ratio of amount in	Free sulfanilamide	1.66	1.66	2.26	1.60	2.56
	Conjugated sulfanilamide	6.29	1.29	0.08	3.79	3.20
100 ml. of corpuscles						
100 ml. of plasma	Total sulfanilamide	2.11	1.56	1.63	1.81	2.63

In Table II data from two individuals, one male and one female, indicate typical variations in distribution between corpuscles and plasma over a period of a number of days. While the picture from Table I is sustained, the data here show that the concentrations of both free and conjugated drug may at times be lower in the corpuscles than in the plasma, and that the ratios of the concentration in corpuscles to that in the plasma for both the free and the conjugated drug may vary greatly from day to day with only small changes in the ratio for the total drug, and that there is not any indication for any accumulation of either free or conjugated drug in the corpuscles.

Distribution of Sulfanilamide in the Blood of Normal Rabbits.—In Table III data are given to show the distribution of sulfanilamide between corpuscles and plasma of the blood of normal rabbits. Three rabbits, Nos. 1, 2, and 4, received 100 c.c. of an aqueous 0.25 per cent solution of the drug by stomach tube twice a day on two successive days, and on the morning of the third day. Specimens of blood were drawn for analysis on the third day three and one-half hours after the administration of the drug. Number 3 represents a composite of blood from three other rabbits. The drug was administered to these rabbits on three consecutive days in the manner indicated above.

TABLE III

DISTRIBUTION OF FREE AND CONJUGATED SULFANILAMIDE BETWEEN CORPUSCLES AND PLASMA IN BLOOD OF NORMAL RABBITS

RABBIT	PLASMA	IN PLASMA			CORPUSCLES	IN CORPUSCLES			RATIO OF AMOUNT IN		
									100 ml. of Corpuscles / 100 ml. of Plasma		
Number	Volumes	Free Sulf.*	Conjugated Sulf.	Total Sulf.	Volumes	Free Sulf.	Conjugated Sulf.	Total Sulf.			
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	Free Sulf.	Conjugated Sulf.	Total Sulf.
1	65.8	65.6	66.4	66.1	34.2	34.3	33.6	33.9	1.13	0.98	1.03
2	65.4	65.5	63.2	63.7	34.6	34.5	36.8	35.2	0.99	1.10	1.08
3†	67.1	63.7	65.6	64.5	32.9	36.3	34.4	35.5	1.13	1.04	1.09
4	71.8	---	---	70.1	28.2	---	---	29.9	---	---	1.08

*Sulf. = Sulfanilamide.

†Number 3 represents composite blood from three rabbits, hourly specimens from each rabbit over an eight-hour period.

Blood specimens were drawn from each of these rabbits at hourly intervals during the entire third day of treatment. Equal amounts of blood from each rabbit were mixed from each drawing, and all eight separate composites were then mixed for a final composite blood for analysis, so that this sample of composite blood should give a general cross section of the distribution of the drug in the blood of three rabbits for a period of eight hours after several days of continuous treatment with the drug. A comparison of volumes per cent of the plasma with per cent of the drug in the plasma and of volumes per cent of the corpuscles with per cent of the drug in the corpuscles indicates that there is not a large preponderance of the drug in the corpuscles. Number 3, which represents a cross section of the blood of three rabbits over an eight-hour period, shows substantially the same distribution as single specimens from individual rabbits. The ratios of concentration in the corpuscles to that in the plasma show the quantitative aspect of the relationship. On an average there appears to be a slight preponderance of total drug in the corpuscles, but this is shown to be a composite of a slight preponderance of the free drug in the corpuscles and of nearly equal distribution of the conjugated drug for rabbit No. 1; of an almost equal distribution of the free drug and a small preponderance of the conjugated drug in the corpuscles for rabbit No. 2; and of a varying degree of preponderance in the corpuscles of both free and conjugated drug for the composite blood of No. 3.

TABLE IV

DISTRIBUTION OF FREE AND CONJUGATED SULFAPYRIDINE BETWEEN CORPUSCLES AND PLASMA IN BLOOD OF MALE PATIENTS

INFECTION	IN WHOLE BLOOD			IN PLASMA			PLASMA	IN PLASMA			CORPUSCLES	IN CORPUSCLES			RATIO OF AMOUNT IN		
	Free S.P.*	Conjugated S.P.	Total S.P.	Free S.P.	Conjugated S.P.	Total S.P.	Volumes	Free S.P.	Conjugated S.P.	Total S.P.	Volumes	Free S.P.	Conjugated S.P.	Total S.P.	100 ml. of Corpuscles / 100 ml. of Plasma		
	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	Free S.P.	Conjugated S.P.	Total S.P.
Meningococcus meningitis	5.00	10.00	15.00	5.72	13.22	18.94	66.4	76.0	87.8	83.8	33.6	24.0	12.2	16.2	0.63	0.28	0.38
Meningococcus meningitis	8.05	2.17	10.22	9.24	2.46	11.70	54.5	62.6	61.8	62.4	45.5	37.4	38.2	37.6	0.67	0.74	0.72
Meningococcus meningitis	8.50	0.95	9.45	9.48	1.33	10.81	61.5	68.6	86.3	70.4	38.5	31.4	13.7	29.6	0.73	0.26	0.67
Meningococcus meningitis	2.89	3.17	6.06	3.27	4.45	7.72	61.6	69.2	85.8	77.9	38.9	30.8	14.2	22.1	0.70	0.26	0.45
Meningococcus meningitis	3.40	1.55	4.95	4.50	1.75	6.25	55.0	73.0	61.9	69.5	45.0	27.0	38.1	30.5	0.45	0.75	0.54
Influenza meningitis	4.09	0.55	4.64	4.02	0.76	4.78	72.5	71.2	100.0	----	27.5	28.8	0.0	----	1.07	0.0	----
Pneumonia	1.71	1.17	2.88	1.75	1.53	3.28	61.8	63.2	81.2	70.5	38.2	36.8	18.8	29.5	0.94	0.38	0.68
Pneumonia and empyema	5.76	0.89	6.65	6.15	1.25	7.40	51.3	54.9	71.9	57.1	48.7	45.1	28.1	42.9	0.87	0.41	0.79
Subacute bacterial endocarditis	7.32	0.88	8.20	7.68	8.83	1.15	71.1	74.6	93.2	76.6	28.9	25.4	6.8	23.4	0.84	0.18	0.75
Osteomyelitis	0.91	1.30	2.21	0.98	1.82	0.84	66.9	72.5	43.0	55.1	33.1	27.5	57.0	44.9	0.75	2.24	2.99

*S.P.=Sulfapyridine.

Distribution of Sulfapyridine in Human Blood In Vivo.—Table IV shows the distribution of sulfapyridine between corpuscles and plasma. The data are arranged in the same manner as in Table I for similar data on the distribution of sulfanilamide. In general, the distribution of sulfapyridine appears to be the opposite of what it is for sulfanilamide, relatively higher concentrations in the plasma than in the corpuscles. This may be seen readily from the per cent values for the drug in the plasma as contrasted with the lower volumes per cent of the plasma, and conversely from the per cent values for the drug in the corpuscles as contrasted with the higher volumes per cent of the corpuscles. The ratios of the concentration in the corpuscles to that in the plasma range from 0.45 to 1.07 for free sulfapyridine, from 0.0 to 2.24 for the conjugated drug, and from 0.38 to 2.99 for the total drug. The ratio is generally higher for the free sulfapyridine than for the conjugated drug. Infrequently it is so high for the free drug as to indicate a slightly higher concentration in the corpuscles than in the plasma. Occasionally an unusually high ratio for the distribution of the conjugated drug may be observed, showing that its concentration may be higher at times in the corpuscles than in the plasma. On the other hand, it is more often entirely absent from the corpuscles. No correlation appears to exist between the type of infection and the distribution between corpuscles and plasma.

TABLE V

VARIATION IN DISTRIBUTION OF FREE AND CONJUGATED SULFAPYRIDINE BETWEEN BLOOD CORPUSCLES AND PLASMA OF TWO PATIENTS

Case 1. Male—Subacute bacterial endocarditis—Streptococcus viridans						
DAYS TREATED		2	3	6	7	8
Ratio of amount in 100 ml. of corpuscles / 100 ml. of plasma	Free sulfapyridine	0.92	0.88	0.72	0.84	0.84
	Conjugated sulfapyridine	0.0	24.9	0.70	----	0.18
	Total sulfapyridine	----	1.24	0.72	----	0.75
Case 2. Pneumonia with empyema—Pneumococcus infection						
DAYS TREATED		1	2	3	4	5
Ratio of amount in 100 ml. of corpuscles / 100 ml. of plasma	Free sulfapyridine	0.95	0.88	0.93	0.75	0.94
	Conjugated sulfapyridine	----	0.87	0.30	1.07	0.38
	Total sulfapyridine	----	0.88	0.80	0.84	0.68

That the distribution of both free and conjugated sulfapyridine may vary considerably from day to day, and especially for the conjugated drug, may be seen in Table V, which represents data from two individuals over a period of eight and five days, respectively. The fluctuation is apparently decidedly greater for the conjugated drug than it is for the free sulfapyridine. While there is one exception in each of the two cases, the general rule appears to be sustained that both free and conjugated sulfapyridine are more highly concentrated in the plasma than in the corpuscles, and that this is especially pronounced for conjugated sulfapyridine.

Distribution of Sulfapyridine in the Blood of Normal Rabbits.—In Table VI data are given to illustrate the distribution of free, conjugated, and total sulfapyridine in the blood of four normal rabbits with reference to the water content of whole blood, plasma, and corpuscles, and also with reference to the

TABLE VI

DISTRIBUTION OF FREE AND CONJUGATED SULFAPYRIDINE BETWEEN CORPUSCLES AND PLASMA IN BLOOD OF FOUR NORMAL RABBITS

RABBIT NUMBER	1			2			3			4		
Plasma, volumes per cent	70.65			61.0			72.8			71.6		
Corpuscles, volumes per cent	29.35			39.0			27.2			28.4		
Blood, Gm. of water per 100 ml.	88.68			85.90			87.81			87.08		
Plasma, Gm. of water per 100 ml.	93.75			94.72			93.80			93.53		
	Sulfapyridine			Sulfapyridine			Sulfapyridine			Sulfapyridine		
	Free	Conjugated	Total	Free	Conjugated	Total	Free	Conjugated	Total	Free	Conjugated	Total
	mg. per	mg. per	mg. per	mg. per	mg. per	mg. per	mg. per	mg. per	mg. per	mg. per	mg. per	mg. per
100 ml. of blood	3.72	7.30	11.02	5.45	17.40	22.85	8.37	3.80	12.17	7.69	8.13	15.82
100 ml. of plasma	4.42	9.61	14.03	6.90	20.70	27.60	10.06	4.11	14.17	9.30	9.50	18.80
100 Gm. of blood water	4.19	8.23	12.42	6.34	20.26	26.60	9.53	4.33	13.86	8.83	9.34	18.17
100 Gm. of plasma water	4.71	10.25	14.96	7.32	21.95	29.27	10.73	4.38	15.11	9.94	10.16	20.10
100 Gm. of corpuscles water	2.61	2.18	4.79	4.34	16.86	21.20	5.35	4.13	9.48	5.12	6.61	11.73
Ratio of amount in 100 Gm. of corpuscle water / 100 Gm. of plasma water	0.55	0.21	0.32	0.59	0.77	0.72	0.50	0.94	0.63	0.52	0.65	0.58
Ratio of amount in 100 ml. of corpuscles / 100 ml. of plasma	0.46	0.18	0.27	0.46	0.59	0.57	0.36	0.73	0.48	0.39	0.49	0.44

distribution on the basis of the volumes of the corpuscles and plasma. Sulfapyridine suspended in water was administered by stomach tube on the basis of one gram per kilogram of body weight per day. Each rabbit received two doses of the drug on the first day—one in the morning and one late in the afternoon—and one on the next morning. Blood specimens were drawn from each rabbit three and a half to four hours after the drug was administered on the second day of treatment. The water content of whole blood and of plasma was obtained by using weighed 1 ml. portions of blood and plasma, warming overnight in an oven at 44° to 45° C., and subsequently bringing to constant weight over calcium chloride in a desiccator. Volumes per cent were obtained from the volumes of corpuscles and plasma of blood, which was centrifuged in calibrated centrifuge tubes

The first part of Table VI gives the volumes per cent of corpuscles and plasma and the grams of water per 100 ml. of blood and per 100 ml. of plasma. These figures are necessary for the calculation of data in the lower part of the table, which gives the levels of the drug in blood and plasma, the concentration of the drug in the water of blood, plasma, and corpuscles, the ratio of the amount in corpuscle water to that in plasma water, and the ratio of the amount in a given volume of corpuscles to that in an equal volume of plasma.

For the sake of comparison the calculation for the concentration of the drug in the water of blood, plasma, and corpuscles was made on the assumption that the drug is held in solution in the water fraction and is not held physically or chemically by components other than water. It can readily be seen from these calculated values that neither free nor conjugated sulfapyridine is distributed between corpuscles and plasma in proportion to the water content. In each of the four rabbits there is only slightly more than half as much free sulfapyridine in 100 Gm. of corpuscle water as in an equal quantity of plasma water. For the conjugated drug the ratio of the content in corpuscle water to that in plasma water varies from 0.21 to 0.94. Apart from the consideration of the water in corpuscles and plasma, the ratios of the amount in corpuscles to that in an equal volume of plasma show for each of the four rabbits a very definite preponderance of both free and conjugated sulfapyridine in the plasma. Thus, there is a definite difference in the distribution between corpuscles and plasma, which cannot be ascribed merely to the difference in water content.

Distribution of Sulfanilamide and Sulfapyridine in Human Blood In Vitro.—In Table VII, a comparison is made of free sulfanilamide and sulfapyridine between corpuscles and plasma of human blood after addition to the blood "in vitro," giving data from three different bloods for each of the drugs. The drugs, dissolved in physiologic saline solution, were added to fresh specimens of human blood. These bloods were then mixed thoroughly, allowed to stand overnight in a cool place, and mixed thoroughly again. Analyses were then made in the usual manner on whole blood and plasma.

It may be seen from the comparison of the per cent of drug in corpuscles and plasma with their volumes per cent that the free sulfanilamide content

tends to be higher in the corpuscles than in the plasma, and that precisely the converse is the case for free sulfapyridine, results which further confirm the preceding data in man.

TABLE VII

SULFANILAMIDE AND SULFAPYRIDINE DISTRIBUTION BETWEEN CORPUSCLES AND PLASMA AFTER ADDITION TO HUMAN BLOOD IN VITRO

SPECIMEN OF BLOOD	PER CENT IN		VOLUMES PER CENT OF	
	CORPUSCLES	PLASMA	CORPUSCLES	PLASMA
		Sulfanilamide		
1	34.1	65.9	23.8	76.2
2	23.6	76.5	23.2	76.8
3	34.7	65.3	24.4	75.6
		Sulfapyridine		
4	24.8	75.2	27.2	72.8
5	26.1	73.9	30.9	69.1
6	27.5	72.5	34.7	65.3

DISCUSSION

Results based on clinical material, on experimental data from rabbits, and on human blood "in vitro" indicate that sulfanilamide, and especially the conjugated form, tends to be distributed in blood in such a way that its concentration is generally higher in the corpuscles than in the plasma. In the case of sulfapyridine the relationship is precisely the opposite. The concentration of sulfapyridine, and especially of its conjugated form, is usually decidedly higher in the plasma than in the corpuscles.

From the experimental data on dogs[2] the conclusion was reached that free sulfanilamide is dispersed throughout the body into the various tissues in direct proportion to their water content. It has been shown that conjugation does not occur in the dog.[5] The data presented here on human and rabbit blood and in an earlier paper[1] indicate that free sulfanilamide and sulfapyridine, as well as their conjugated forms, are distributed unequally between corpuscles and water and in a manner out of harmony with the idea of distribution in proportion to water content. A similar conclusion was reached recently by Size[6] in a report on sulfanilamide in human blood. The idea I expressed[1] and the one recently expressed by Size[6] that sulfanilamide may be loosely bound physically or chemically by something in the corpuscles may not be an adequate explanation for the difference in concentration in corpuscles and plasma. It certainly is not for sulfapyridine. It may be that the explanation is to be found chiefly in the difference in the rate of diffusion or absorption through the cell membrane of the corpuscles and other cell and tissue membranes.

While not reported in this paper, the clearance rates and ratios for free and conjugated sulfanilamide were determined for many individuals under treatment, and in a number of cases such determinations were made daily during the entire period of treatment. The clearance rate was generally found to be much higher for conjugated sulfanilamide than for the free drug, confirming previous reports.[1, 7, 8] This difference is probably due to a difference in reabsorption by the tubules, since "in vivo" synthesis of the conjugated

form of the drug does not occur in the kidney, but appears to be limited to the liver.[9, 10] The picture for the conjugated form may be complex, since evidence has been given recently that conjugation may occur with glycuronic acid.[11] However, whatever the form of combination, the facts about relative clearance are unaltered. It seems likely, therefore, that concentrations low in the plasma with reference to concentration in the corpuscles or the entire absence from the plasma may be largely explained by a difference in the rate of elimination by the kidney and passage out of the tissues and the corpuscles into the plasma. This view finds further support from the fact that the very low concentration, in, or the entire absence from, the plasma of free and conjugated sulfanilamide is generally found when the total concentration in the blood is also low. The explanation for the distribution of sulfapyridine, which is precisely opposite to that of sulfanilamide, may also lie chiefly in differences in diffusion or absorption through living membranes. Data for the clearance rates of free and conjugated sulfapyridine are not at hand, but it is a well-known fact that sulfapyridine is much less soluble than sulfanilamide and that acetylsulfapyridine is decidedly less soluble than free sulfapyridine. Nevertheless, differences in diffusion rates through living membranes may not be adequate to explain the slow release of all of the drug from corpuscles in the blood[1] and from other tissues,[12] nor does it appear to explain satisfactorily the distribution of free sulfanilamide and sulfapyridine between corpuscles and plasma of human blood "in vitro."

CONCLUSIONS

(1) In "in vivo" studies sulfanilamide, and especially conjugated sulfanilamide, are generally found in higher concentration in the corpuscles than in the plasma of human and rabbit blood.

(2) In "in vivo" studies sulfapyridine, and especially conjugated sulfapyridine, are generally found in higher concentrations in plasma than in the corpuscles of human and of rabbit blood.

(3) Addition of sulfanilamide and of sulfapyridine to human blood "in vitro" results in distribution between corpuscles and plasma in the same manner as stated for "in vivo" studies under (1) and (2).

(4) Relative concentrations of sulfanilamide and of sulfapyridine and of their conjugated forms in corpuscles and plasma vary considerably from day to day, and there does not appear to be any tendency to accumulation of any form of either drug in either corpuscles or plasma.

(5) Sulfanilamide and sulfapyridine and their conjugated forms are not distributed between corpuscles and plasma of human and rabbit blood in proportion to the water content of these blood components.

(6) The type of infection or the influence of sex does not seem to have any effect upon the distribution of sulfanilamide or sulfapyridine between corpuscles and plasma of human blood.

I wish to express my appreciation to Dr. W. A. Kreidler for assistance in the animal experiments; to the various members of the Jefferson Hospital staff who have made clinical material available; to Dr. Tracey Cuttle for the clinical material on meningitis cases; and to Professor George R. Bancroft for encouragement and suggestions.

REFERENCES

1. Hansen, Lorenz: Sulfanilamide Concentration and Distribution in Blood and Urine in Sulfanilamide Therapy for Gonococcic Infections in Men, J. Pharmacol. & Exper. Therap. **65:** 372, 1939.
2. Marshall, E. K., Jr., Emerson, Kendall, Jr., and Cutting, W. C.: Distribution of Sulfanilamide in the Organism, J. Pharmacol. & Exper. Therap. **61:** 196, 1937.
3. Marshall, E. K., Jr.: The Determination of Sulfanilamide in Blood and Urine, J. Biol. Chem. **122:** 263, 1937.
4. Marshall, E. K., Jr., and Litchfield, J. T., Jr.: The Determination of Sulfanilamide, Science **88:** 83, 1938.
5. Marshall, E. K., Jr., Emerson, Kendall, Jr., and Cutting, W. C.: Para-Aminobenzenesulfonamide; Absorption and Excretion. Method of Determination in Urine and Blood, J. A. M. A. **108:** 953, 1937.
6. Size, Herbert S.: Distribution of Sulfanilamide and Acetyl-Sulfanilamide Between Cells and Extracellular Fluid, Proc. Soc. Exper. Biol. & Med. **40:** 451, 1939.
7. Marshall, E. K., Jr., Emerson, Kendall, Jr., and Cutting, W. C.: Renal Excretion of Sulfanilamide, J. Pharmacol. & Exper. Therap. **61:** 191, 1937.
8. Stewart, J. D., Rourke, G. M., and Allen, J. G.: Excretion of Sulfanilamide, J. A. M. A. **110:** 1885, 1938.
9. Klein, J. R., and Harris, J. S.: The Acetylation of Sulfanilamide in Vitro, J. Biol. Chem. **124:** 613, 1938.
10. Stewart, John D., Rourke, G. Margaret, and Allen, J. Garrett: Acetylation of Sulfanilamide, Surgery **5:** 232, 1939.
11. Scudi, J. V., Ratish, H. D., and Bullowa, Jesse G. M.: Increased Glycuronate Excretion Following Administration of Sulfapyridine, Science **89:** 516, 1939.
12. Pinto, Sherman S.: Excretion of Sulfanilamide and Acetyl-Sulfanilamide in Human Milk, J. A. M. A. **111:** 1914, 1938.

A NOTE ON THE LEVEL OF GLUCOSE AND OF NONFERMENTABLE REDUCING SUBSTANCES IN THERAPEUTIC INSULIN SHOCK*

W. H. Forbes and T. Czarski, Baltimore, Md.

IN THE literature published prior to 1933 or 1934 it is generally stated that hypoglycemic shock occurs when the blood sugar falls below 45 mg. per 100 c.c. of blood.[1] This figure refers to the blood sugar as determined by one of the methods which are in common use in clinical laboratories and not to be "true glucose" or, more precisely, the fermentable reducing substances in the blood. The fermentable reducing substances, obtained from the difference in the reducing power of the blood filtrates before and after fermentation, run from 10 to 30 mg. lower than the blood sugars as commonly measured.[1, 2] These 10 to 30 mg. represent the reduction due to nonfermentable reducing substances, most of which come from the corpuscles. It has been found that the amount of these substances is independent of the level of the blood sugar from 50 mg. to 550 mg.,[1, 3, 4] and our observations show that even when the glucose is pushed down below 10 mg. the nonfermentable reducing substances remain unchanged in amount (Table I). This table also shows that though these substances do not change with changes in the level of glucose, they do change markedly with rela-

*From the Department of Biological Chemistry of the Johns Hopkins Medical School, and the Laboratory of Internal Medicine of the Henry Phipps Psychiatric Clinic, Baltimore.

Received for publication, June 22, 1939.

†Throughout this paper the figures will refer to "milligrams per 100 c.c. of blood," but this expression will be abbreviated to "mg."

TABLE I

"BLOOD SUGAR" AND "TRUE GLUCOSE" IN SCHIZOPHRENIC PATIENTS WITH THERAPEUTIC INSULIN SHOCK

DATE	SUBJECT AND TREATMENT	METHOD A REVISED FOLIN-WU "LAKED" METHOD		METHOD B "UNLAKED" FOLIN METHOD		"True Glucose," i.e., average of the values obtained by Method A and Method B	CONDITION OF SUBJECT WHEN SAMPLE WAS TAKEN
		"Blood Sugar," i.e., total reducing substances in mg. per 100 c.c. of blood	"True Glucose," i.e., fermentable reducing substances in mg. per 100 c.c. of blood	"Blood Sugar," i.e., total reducing substances in mg. per 100 c.c. of blood	"True Glucose," i.e., fermentable reducing substances in mg. per 100 c.c. of blood		
	Males						
Jan. 14	Bl. Before insulin	83	59	67	60	60	Fasting and resting
	Bl. 3 hr. after 45 units	59	35	39	32	34	Perspiring but not comatose
	Ma. Before insulin	80	58	63	55	57	Fasting and resting
	Ma. 3 hr. after 140 units	50	24	29	23	24	Perspiring but not comatose
Feb. 4	Bl. Before insulin	106	90	91	87	89	Fasting and resting
	Bl. 3½ hr. after 140 units	48	32	24	25	29	Restless, not comatose
	Ma. Before insulin	108	94	88	86	90	Fasting and resting
	Ma. 3¾ hr. after 160 units	38	23	23	18	20	Comatose for 1 hr.
	Th. Before insulin	101	89	89	87	88	Fasting and resting
	Th. 3¾ hr. after 75 units	33	21	24	21	21	Restless, not comatose
May 3	H. 2 hr. after 55 units	22	12	11	7	10	Light coma
	H. 3½ hr. after 55 units	20	8	6	2	5	Deep coma
	S. 2 hr. after 55 units	23	12	12	7	10	Light coma
	S. 3½ hr. after 55 units	21	8	10	6	7	Deep coma

TABLE II

"TRUE GLUCOSE," I.E., FERMENTABLE REDUCING SUBSTANCES IN SCHIZOPHRENIC PATIENTS WITH THERAPEUTIC INSULIN SHOCK

DATE	SUBJECT AND TREATMENT	METHOD A. CALCULATED "TRUE GLUCOSE" (= BLOOD SUGAR − 11 MG.)	METHOD B. CALCULATED "TRUE GLUCOSE" (= BLOOD SUGAR − 4 MG.)	AVERAGE "TRUE GLUCOSE"	CONDITION OF SUBJECT WHEN SAMPLE WAS TAKEN
	Males				
May 4	H. Before insulin	86	87	87	Fasting and resting
	H. 2 hr. after 55 units	11	14	13	Light coma
	H. 4 hr. after 55 units	7	6	7	Deep coma
	S. 2½ hr. after 55 units	15	15	15	Perspiring but not comatose
	S. 3½ hr. after 55 units	12	8	10	Light coma
May 5	H. 3½ hr. after 55 units (fluoride)	12	13	13	Deep coma
	H. 3½ hr. after 55 units (oxalate)	11	9	10	Deep coma
	3½ hr. after 55 units	13	7	9	Deep coma
	Females				
Aug. 31	Pu. 2 hr., 40 min. after 50 units		9		Deep coma
	Ha. 2 hr., 45 min. after 70 units		4		Moderate coma
	Si. 2 hr., 50 min. after 40 units		4		Moderate coma
	Re. 2 hr., 55 min. after 50 units		5		Light coma
	Mo. 3 hr. after 40 units		8		Deep coma
	Be. 4 hr. after 35 units		10		Light coma, recovering
	Ho. 4 hr., 5 min. after 50 units		10		Light coma

tively slight changes in the methods of determining blood sugar. For example, on January 14, the day of our first experiment, the reagents were old. On finding that the nonfermentable reducing substances in the filtrates were 24 mg., fresh precipitating reagents were prepared with the result that in all subsequent experiments this figure was 13 mg. ±3. The fact that not only the methods of precipitation, but also the methods of measuring the reduction, differ in their specificity, makes it difficult to compare the results obtained by different observers. Depending upon the methods used, the "blood sugar" will exceed the fermentable reducing substances by as little as 2 mg., or as much as 35 mg. The Folin-Wu method, used upon laked filtrates precipitated with tungstic acid, measures as "sugar" approximately 15 to 25 mg. of nonfermentable reducing substances. If 20 mg. is subtracted from the figure of 40 to 50 mg., which is given in the earlier literature, one obtains a figure between 20 and 30 mg. as the probable value of the fermentable reducing substances when shock begins. This agrees, in a general way, with later work, but values outside these limits are frequently reported. Recent work on human beings and animals has shown that during coma it may be much lower: zero in animals[5] and 5 mg. in an infant.[6]

The current treatment of schizophrenia by insulin shocks has afforded an opportunity to follow the total and fermentable reducing substances in the blood

of adults as the glucose is pushed down to levels which produce coma. While it is not certain that normal individuals would have become comatose at the same levels of blood sugar as did these patients, there is, on the other hand, little, if any, reason to believe that the level of coma is altered in schizophrenia. The interest of our observations lies not so much in the level at which coma begins as in the very low levels to which the glucose can fall without causing the death of the individual.

METHOD

Blood was drawn from the arm vein of a fasting patient before the injection of insulin and once or twice thereafter, usually at two and one-half or three and one-half hours. Oxalate was used as an anticoagulant in most cases; fluoride in a few. The blood was put in the icebox immediately and precipitated as soon as was convenient, usually within an hour of drawing the last sample. Two methods of precipitation were used, the ordinary Folin-Wu,[7] and the "unlaked" Folin.[2] The reducing substances were measured by the revised Folin-Wu method.[8] The filtrates were then brought to about pH 6.5 and fermented for fifteen minutes with washed yeast at 35° C. and the after-fermentation values were determined. Blanks and controls were done, using the same amount of yeast mixed with water for the blanks and with glucose solution for the controls. The controls showed that the fermentation was complete in less than ten minutes.

The results are given in Table I. The "true glucose" is determined by subtracting the yeast blank from the after-fermentation figure, thereby getting the true after-fermentation value (i.e., the nonglucose reducing substances), and subtracting this difference from the "blood sugar" (i.e., total reducing substances) of the unfermented filtrate.

It has been found[2, 4, 9] that the "unlaked" method is fairly specific for the fermentable reducing substances and includes, on the average, only about 3 or 4 mg. of nonfermentable reducing substances. This is confirmed by the data in Table I. Hence one may calculate the "true glucose" with a fair degree of accuracy by subtracting 4 from the blood sugar, as measured by the unlaked method. The same thing can be done with the laked method, though the amount of nonfermentable reducing substances is larger and less certain, averaging in our experiments (except the first) just over 13 mg., as mentioned above. Table II gives the fermentable reducing substances in the later experiments, calculated from the observed blood sugars by subtracting 14 for the laked method and 4 for the unlaked. The latter half of the table gives the values obtained from 7 female patients from whom only one blood was taken.

RESULTS

None of those whose fermentable reducing substances were over 20 mg. were comatose, and all but 2 of those under 20 mg. were comatose, but the number of individuals is too small (18 insulin shocks given to 12 patients) to assign a very definite figure to the level at which coma begins. Furthermore, there was no clear relationship between the apparent depth of the coma and the blood

sugar. In general, the samples were taken at the times when the blood sugar was thought to be at its lowest point, but there are no means of knowing whether we succeeded in getting the lowest point. No untoward aftereffects were seen even when the glucose fell below 10 mg. and remained there for an hour or so, but in these subjects slight injuries to the central nervous system might well pass unnoticed, especially if they were diffuse. This is very near the level at which the damage done to the nervous tissue is so serious and widespread as to prove fatal.

SUMMARY

The figures in Table I confirm other workers[2, 4, 9] in showing that the glucose may be determined with a probable error of ±2 mg. by subtracting 4 mg. from the blood sugar, as determined upon an unlaked filtrate by the revised Folin-Wu method.

In therapeutic insulin shock in schizophrenic patients the glucose (i.e., fermentable reducing substances) may fall below 10 mg. per 100 c.c. of blood and remain there for periods up to an hour without doing harm of an extent sufficient to be obvious in these subjects. The nonfermentable reducing substances do not change in amount as the blood sugar falls.

We wish to thank Drs. M. Hayman and R. Wilmanns of the Springfield State Hospital for help and facilities in obtaining these samples.

REFERENCES

1. Peters, J. P., and Van Slyke, D. D.: Quantitative Clinical Chemistry, Baltimore, 1932, Vol. I, pp. 104 and 105, Williams & Wilkins Co.
2. Folin, O.: Unlaked Blood as a Basis for Blood Analysis, J. Biol. Chem. **86:** 173, 1930.
3. Folin, O., and Svedberg, A.: The Sugar in Urine and in Blood, J. Biol. Chem. **70:** 405, 1926.
4. Andreen-Svedberg, A.: On the Distribution of Sugar Between Plasma and Corpuscles in Animal and Human Blood, Skandinav. Arch. f. Physiol. **66:** 113, 1933.
5. Dotti, L. B., and Hrubetz, M. C.: The True Blood Sugar Level in Insulin Shock and Convulsions, J. Biol. Chem. **113:** 141, 1936.
6. Hartmann, A. F., and Jandon, J. C.: Hypoglycemia, J. Pediat. **11:** 1, 1937.
7. Folin, O., and Wu, H.: A System of Blood Analysis, J. Biol. Chem. **38:** 81, 1919.
8. Folin, O.: Two Revised Copper Methods for Blood Sugar Determination, J. Biol. Chem. **82:** 83, 1929.
9. Unpublished work by one of the authors (Forbes, 1934).

VITAMIN C STUDIES

I. The Effect of Vitamin P (Citrin) on Vitamin C Deficient Guinea Pigs*

Lawrence E. Detrick, Ph.D., Max S. Dunn, Ph.D., W. L. McNamara, M.D., and M. E. Hubbard, M.D., Los Angeles, Calif.

AN EXTRACT of paprika or lemon juice has been reported to be more effective in the clinical treatment of increased vascular permeability than crystalline vitamin C. Therapeutic potency for the treatment of purpuric conditions was observed in the flavanol glucoside "citrin" fraction of lemon juice. Citrin healed the increased capillary permeability in 3 patients with vascular purpura, but had little, if any, effect on 4 patients with thrombopenic purpura. It exerted a moderate effect in 7 patients with infectious disease, one person with myxedema and 2 persons with diabetes.[1]

Experimentally citrin was found to decrease the number of hemorrhages in scorbutic guinea pigs and prolonged the survival period from 28.5 days for the negative control group to 44 days for the animals given 1 mg. of citrin daily.[2] These findings were not confirmed by the investigations of Zilva[3] nor could they be maintained when the work was repeated by one of the original investigators.[4]

Citrin, which was originally considered a mixture of flavanones, has been resolved into its two chief components, hesperidin and eriodictyol glucoside.[5] Eriodictyol glucoside is demethylated hesperidin and is frequently referred to as eriodictin.[6] It is found in higher concentration in lemons than in oranges.

In this experiment we proposed to study the alleged activity of citrin in scorbutic guinea pigs as a preliminary investigation to its use in the clinical purpuras. A more detailed discussion of the chemistry of isolation, color reactions, and solubilities of these citrin preparations will be found in a separate paper to be contributed by the Research Department of the California Fruit Growers Exchange.

EXPERIMENTAL

Four groups of experimental animals (20 guinea pigs each) were used to test the activity of citrin preparations 3, 4, 5, and 6 (Table I) during the months of November, January, March, and June. All animals were given a Sherman-Lamar-Campbell vitamin C deficient diet. Each experimental group was subdivided into four subgroups: Group A, 4 animals, was given 4 c.c. of orange juice daily for an adequate supply of vitamin C and no citrin; Group B,

*From the Department of Chemistry, University of California, and the Veterans Administration Facility, Los Angeles.

Received for publication, June 26, 1939.

4 animals, received the vitamin C deficient diet only; Groups C and D, 6 animals each, were administered different citrin preparations. Citrin in amounts equivalent to that isolated from 100 c.c. of lemon juice was administered daily either orally or intraperitoneally. The animals were weighed twice a week. When the physical condition reached the stage where death seemed imminent, they were killed and studied for the extent and severity of hemorrhagic lesions.

TABLE I

PROPERTIES OF VARIOUS FRACTIONS OF VITAMIN P
Isolated by C. W. Wilson

PREPARATION NUMBER	COLOR	FORM	MELTING POINT	BORIC ACID REACTION	Fe Cl_3 REACTION	ASH %	Ba	LEAD
1. B-5837 (Hesperidin)	White	Microscopic needles	257-9 (Decomp.)	None	Weak	None	None	None
2. B-5838	Pale yellow	Microscopic needles	254-6 (Decomp.)	Slight	None	0.25	Small	None
3.* B-5852	Bright yellow	Short microscopic needles	241-45 (Decomp.)	+	Emerald green	0.40	Small	None
4.* B-5917 W.S.	Reddish orange glass before vacuum drying	–	–	++	Dirty green, brown with excess	2.1	Large	None
5.* B-6226 W.S.	Greenish brown	Chiefly amorphous few crystals	–	++	Dirty green	9.65	–	–
6.* B-6229	Golden yellow	Amorphous hygroscopic	–	++	Dirty green	10.70	–	–

*Preparations tested.
W.S. = Water soluble.

Citrin preparations 3 and 4, administered by mouth or injected intraperitoneally during the months of November, January, or March, did not prolong the life or ameliorate the hemorrhagic symptoms of scurvy. Twenty-five per cent of these animals, other than the positive control groups that were given orange juice, developed ulcerous lesions in the stomach. The stomach lesions were of two types; one was round, with a clear gelatinous edge, and varied in size from a pin point to the size of the head of a common pin; and the other was moth-eaten in appearance, irregular in shape, occasionally bifurcated, with opaque white edges.

Citrin preparations 5 and 6 injected intraperitoneally into guinea pigs during the month of June did not affect the hemorrhagic diathesis of scurvy. Water-soluble preparation 5 ameliorated the physical deterioration of the animals during the last days of the experiment. Preparation 6 showed no tendency to prolong the life of the animals. Fig. 1 gives a graphic illustration of the effect of preparations 5 and 6 upon the body weight of the June animals.

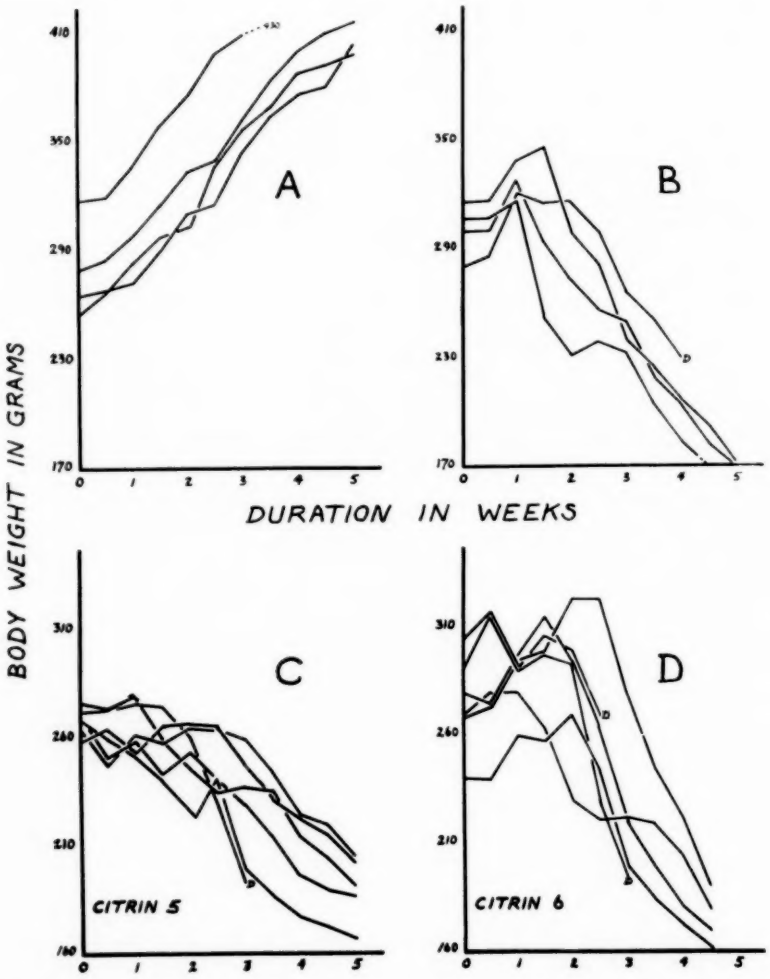


Fig. 1.—Body weight curves for June animals. *D* denotes death of animal.

COMMENT

Our observations are in agreement with those of other investigators,[3, 4, 6] in that citrin neither decreased the hemorrhagic condition of scurvy nor prolonged the life of the animals. In only one group (Fig. 1, Group C) did citrin improve the physical condition of scorbutic guinea pigs. Since stomach lesions appeared in 20 per cent of the vitamin C deficient animals run during the months of November, January, and March, and not a single stomach lesion appeared in the June experiment, we conclude that winter and summer animals differ in some unknown manner. This unknown factor, probably dietary, protected vitamin C deficient guinea pigs from developing stomach lesions and acted with citrin preparation 5 to improve the general physical condition, but had no effect upon the hemorrhagic diathesis of scurvy.

Clinically, citrin continues to be found effective in capillary wall permeability but not active in thrombopenia. Further investigations are under way in this field.

CONCLUSION

Citrin preparations isolated by the technique of Armentano and others,[1] though different in minor respects (details to be published by C. W. Wilson and co-workers), did not decrease the hemorrhagic lesions of the scurvy syndrome or prolong the life of the scorbutic guinea pigs.

We are indebted to C. W. Wilson, of the Research Department of the California Fruit Growers Exchange, for the careful preparation and analysis of the citrin used in this investigation (Table I).

REFERENCES

1. Armentano, L., Bentsáth, A., Beres, T., Rusznyák, S., und Szent-Györgyi, A.: Über den Einfluss von Substanzen der Flavongruppe auf die Permeabilität der Kapillaren Vitamin P, Deutsche med. Wchnschr. **62:** 1325, 1936.
2. Bentsáth, A., Rusznyák, S., and Szent-Györgyi, A.: Vitamin Nature of Flavones, Nature, London **138:** 798, 1936.
3. Zilva, S. S.: Vitamin P, Biochem. J. **31:** 915, 1937.
4. Bentsáth, A., and Das, N. B.: Über den Vitamin P-Test, Ztschr. f. physiol. Chem. **247:** 258, 1937.
5. Bruckner, V., and Szent-Györgyi, A.: Chemical Nature of Citrin, Nature, London **138:** 1057, 1936.
6. Szent-Györgyi, A.: Methoden zur Herstellung von Citrin, Ztschr. f. physiol. Chem. **255:** 126, 1938.

URINARY PROLAN EXCRETION DURING A MENSTRUAL CYCLE*

B. B. Bagby, Jr., M.D., Catawba Sanatorium, Va.

RECENTLY we reported a clinical method for determining the urinary prolan excretion during the menstrual cycle of a normal human female, utilizing various accepted techniques.[1] This paper reports the urinary prolan excretion of a normal woman during one menstrual cycle.

Various investigators have found the pituitary gonadotropic hormone (F. S. H.) present in blood and urine for a few days of the menstrual cycle in comparatively large amounts, whereas it is practically absent the rest of the time. This increased amount usually occurs in the middle of the cycle and is believed to be an indication of ovulation; it does not necessarily occur at a regular time in the cycle. D'Amour[2] and co-workers have shown that the peak may vary from early to late in the period and that in some subjects there may be two separate peaks.

In this experiment both the uterine and the ovarian weights were determined. This was done because Cartland and Nelson[3] have shown that the uterine weight is a more sensitive indicator for small amounts of prolan, although some believe that if dependence is placed in either ovarian or uterine weights, positive results may be missed. Drips and Osterberg[4] use the microscopic appearance of the ovary as an index, but I am not sure that this is necessarily better.

*From the Department of Clinical Pathology, University of Virginia.
Received for publication, June 28, 1939.

I did not attempt to use the two day—6 injections technique of Aschheim and Zondek because I had found, from my previous experiment,[1] that the toxicity of the urinary concentrate due to potassium salts[5] caused rats to die during the course of the experiment. Instead I used the method of Heller,[6] in which urinary concentrate is dissolved in 9 c.c. of water, giving 1 c.c. per dose and 2 doses a day subcutaneously to 21-day-old female rats. The animals were killed on the morning of the sixth day.

The subject was a nurse, aged 19 years, with a normal menstrual history. She had never experienced any intermenstrual pains.

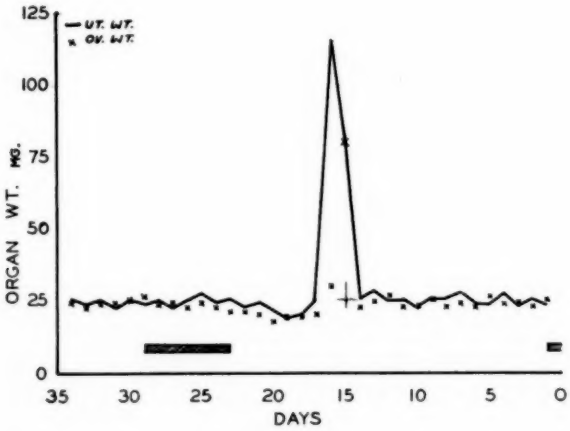


Fig. 1.

In order to make the collecting of urine less tedious I used only the first morning specimen of urine instead of the twenty-four-hour specimen, as suggested previously.[1] This specimen was collected in a bottle containing a small amount of chloroform as a preservative. The urine was then kept on ice until the afternoon or evening when it was acidified to litmus with acetic acid if necessary and then filtered. Four parts of ethyl alcohol were added, the mixture thoroughly mixed and placed in the icebox for at least twenty-four hours. The supernatant fluid was decanted and the precipitate collected by centrifuging. It was then washed twice in ether with careful mixing and dried in air. The precipitate was placed in a small section dish and kept in the icebox until time for injection.

Twenty-four hours before use this precipitate was dissolved in approximately 10 c.c. of distilled water. Ten cubic centimeters were used because the precipitate retained some of the water; I found that this amount of water gave me 9 c.c. of solution to inject. Before injection the remaining precipitate was centrifuged and the prolan solution was decanted. The first injection was given on the afternoon of the first day, and then an injection was made every morning and afternoon until a total of nine injections had been given. On the morning of the sixth day the rats were killed with ether, the uterus was stripped clean, and the fluid, if any, was pressed out on a filter paper moistened with normal saline. The uterus was then immediately weighed in a weighing bottle. Both ovaries were then freed of fascia and weighed together.

It is of some interest to note that none of the rats died during the course of the experiment. The results are shown in Fig. 1. The peak occurred on the fifteenth and sixteenth days prior to the next period (the seventh and eighth days following the cessation of the previous period).

Even though there may be an increase in the excretion of estrogen at the time of the peak, which may cause the increase in the weight of the uterus, the definite increase in the size of the ovaries at this time shows increase in the prolan excretion.

These results again indicate that it is probably unnecessary to take the trouble to weigh the ovaries, since the uterine weight appears to be a far more sensitive indicator. It seems improbable that any estrogenic hormone could be influencing the result, since the uterine weight in this patient and in my previous one[1] increases only when the ovarian weight increases. If estrogenic hormones were coming through the concentration process, we could expect the uterine weight to increase at other times, especially just before menstruation, unaccompanied by an increase in the ovarian weight.

The cost of the experiment, not including the time and overhead, was approximately $20.00. This is still too expensive a procedure to warrant general clinical application.

SUMMARY

The urinary prolan excretion of a normal woman was determined daily for one month, on the first morning specimen, using ovarian and uterine weights of immature rats as indicators. Prolan was found on the fifteenth and sixteenth days prior to the next menses.

I wish to express my indebtedness to Dr. W. E. Bray and to Dr. R. J. Main for their assistance and criticism.

REFERENCES

1. Bagby, B. B., Jr., and Main, R. J.: The Determination of Prolan Excretion During the Menstrual Cycle, Virginia M. Monthly **65**: 756, 1938.
2. D'Amour, F. E., Funk, Dorothy, and Liverman, Helen: Daily Gonadotropic Determinations During Forty Complete Menstrual Cycles, Am. J. Physiol. **123**: 47, 1938.
3. Cartland, G. F., and Nelson, J. W.: The Bioassay of Mare Serum Hormone, Am. J. Physiol. **122**: 201, 1938.
4. Drips, Della G., and Osterberg, Arnold E.: An Evaluation of the Frank Method for the Determination of Prolan (Gonadotropic Principle) in the Urine of Nonpregnant Women, Endocrinology **23**: 703, 1938.
5. Main, Rolland J.: Mineral Salts as Toxic Factors in Urinary Prolan Concentrates, Endocrinology **24**: 523, 1939.
6. Heller, Carl G., Lausen, Henry, and Sevringhaus, Elmer L.: The Immature Rat Uterus as an Assay End-Point for Gonadotropic Substances, Am. J. Physiol. **121**: 364, 1938.

THE EFFECT OF SULFANILAMIDE ON THE CROSS MATCHING OF BLOOD*

Preliminary Report

Sidney Kreinin, M.D., Brooklyn, N. Y.
With the Technical Assistance of
Frances A. Hamblen, B.S., and Lucy Porcelli, B.A.

THOUGH many and varied are the side reactions to sulfanilamide, there have been no previous reports in the literature of the effect of the drug on the cross matching of blood. It has been our experience that the blood of patients who had been given sulfanilamide was difficult to cross match.[1] Communication with the medical department of one of the large pharmaceutical houses reveals that they have received many requests concerning such an effect of the drug on the cross matching of blood, but that no study was available.[2]

It was, therefore, decided to determine the effect, if any, of the drug upon the cross matching of blood.

Upon admission to the hospital the blood of each patient was typed and cross matched. A complete blood study—red blood cell and white cell counts, sedimentation time, and blood chloride determination—was also done. Sulfanilamide 15 gr. to 20 gr. with sodium bicarbonate was given every four hours for a period ranging from forty-eight to seventy-two hours. Following this a sulfanilamide concentration test (Marshall's method)[3] was done and the patient was recross matched. Again the sedimentation time and the blood chlorides were determined.

COMMENT

Although the number of cases reported is too few for any final conclusions to be drawn, certain facts are apparent.

Sulfanilamide per se does not disturb the cross matching of blood.

Accepting the sulfanilamide concentration test as an indication of the ability of the patient to absorb sulfanilamide, it was noted that there was a wide variation in the rate of absorption and excretion of the drug among the patients. One patient who had received 120 grains of sulfanilamide exhibited 15 mg. per 100 c.c. of blood while another patient, under the same dosage, had none in the blood. A patient who had received 300 grains of the drug had a concentration of 3 mg. while one receiving 100 grains had a concentration of 18 mg.

*From the medical and surgical services of Dr. L. T. Fricke and Dr. G. Kasper, respectively, at the Swedish Hospital.

The Winthrop Chemical Co. supplied the sulfanilamide (Prontylin) used in this study. The blood was furnished by Mixon's Blood Donor Agency.

Received for publication, June 30, 1939.

TABLE I

NO.	SUBJECT	SEX	AGE	DISEASE OR CONDITION	BEFORE SULFANILAMIDE					AFTER SULFANILAMIDE				
					BLOOD TYPE	CROSS MATCHING	HB	BLOOD CHLORIDES	SEDIMENTATION FALL IN 60 MIN	TOTAL AMOUNT SULFANILAMIDE	SULFANILAMIDE CONCENTRATION TEST	BLOOD CHLORIDES	SEDIMENTATION FALL IN 60 MIN.	RESULT OF CROSS MATCHING
									mm.	gr.	mg.		mm.	
1	C. L.	♂	54	Fracture left hand	A	Good	98	396	20	180	10	462	23	Good
2	C. M.	♀	39	Cardiac neurosis	B	Good	80	396	11	240	16	561	21.5	Good
3	R. S.	♀	28	Rheumatic polyarthritis	O	Good	84	462	28	300	3	478	24	Good
4	N. R.	♂	16	Acute appendicitis	A	Good	94	330	15.5	120	0	360	10	Good
5	J. A.	♀	38	Myositis	A	Good	100	478	3	120	15	396	4	Good
6	P. O.	♀	18	Acute rheumatic fever	O	Good	88	825	22	150	7.6	132	26	Good
7	P. A.	♀	59	Cirrhosis liver	O	Good	80	332	26	195	10	660	1.5	Good
8	B. S.	♀	28	Rheumatic fever	O	Good	68	330	28.5	165	13	511	28	Good
9	W. E.	♀	39	Hyperthyroidism	O	Good	62	363	20	210	17	610	29	Bad on patients' serum—donors' cells Good on patients' cells—donors' serum
10	P. M.	♀	29	Appendicitis	AB	Good	78	330	0	120	7		6	Good
11	B. H.	♂	23	Appendicitis	O	Good	80	396	1	160	8	544	3	Good
12	B. C.	♀	28	Pyelocystitis	B	Good	66	363	8	90	8		12	Good
13	W. R.	♂	49	Urethral stricture	O	Good	84	363	17	100	18	346	20	Good
14	V. E.	♀	49	Chronic polyarthritis	A	Good	73	544	11	120	13	528	25	Good
15	S. C.	♂	52	Hypertrophied prostate	A	Good	82	455	16	160	10	292	30	Good
16	S. L.	♀	23	Upper respiratory infection	O	Good	98	412	3	160	4	380	1	Good
17	L. A.	♀	21	Acute appendicitis	A	Good	78	577	8	120	9	445	6	Good
18	G. B.	♀	33	Influenzal pneumonia	O	Good	72	528	4	135	4	396	27	Good
19	M. J.	♂	43	Cirrhosis liver	B	Good	60	511	17	200	5	412	5	Good
20	H. M.	♀	70	Diverticulosis	A	Good	84	346	13	120	0	363	4	Good
21	W. H.	♀	40	Chronic appendicitis and salpingo-oophoritis	B	Good	90	412	23	90	6	396	25	Good
22	D. G.	♂	19	Influenza	B	Good	92	495	2	240	4	495	2	Good
23	W. H.	♀	48	Influenza	B	Bad	82	561	20	180	12	627	10	Bad on patients' cells—donors' serum Good on patients' serum—donors' cells
24	G. M.	♀	58	Auricular fibrillation	A	Bad on patients' serum—donors' cells Good on patients' cells—donors' serum	95	464	12	165	8	760	2	Good

In only two cases was difficulty encountered in the cross matching of blood.

In one case (No. 9) the patient's serum agglutinated the cells of the donor. There was no agglutination when the patient's cells and the donor's cells were mixed.

In another case (No. 23) the donor's serum agglutinated the patient's cells. There was no agglutination when the patient's serum and the donor's cells were mixed.

In Case 24 the patient's serum agglutinated the donor's cells before the administration of sulfanilamide. Following the administration of the drug, no agglutination was noted.

It seems, then, that the inability to cross match blood following the administration of sulfanilamide is due to changes in the blood brought about by the disease requiring the use of sulfanilamide rather than to the drug itself.

REFERENCES

1. Kreinin, S.: Pneumococcic Meningitis With Recovery, Arch. Otol. **29**: 371, 1939.
2. Personal communication to author.
3. Marshall, E. K., Jr., Emerson, K., and Cutting, W. C.: Para-Aminobenzenesulphonamide: Absorption and Excretion. Method of Determination in Urine and Blood, J. A. M. A. **108**: 953, 1939.

5722 Fourth Avenue

RELATION OF LYMPHOCYTES TO THE VIRULENCE OF PNEUMOCOCCI TYPES III AND VII*

L. A. Turley, M.A., Ph.D., and Thomas F. Dougherty, M.A.
Oklahoma City, Okla.

IT IS rather remarkable that our knowledge of the lymphocyte, especially its function, is so meager, fragmentary, theoretical, and speculative, especially when we consider that the total mass of lymphocytic tissue in the body is greater than that of all the other hemocytic tissue combined, that the lymphocytes are the third most numerous cells in the circulating blood, that lymphocytes are present in almost every inflammatory lesion, toxic malignant growth, and toxic thyroid; and that the lymphocyte is the most universal cell in circulating fluids. The experimental work that has been done bearing on the function of the lymphocyte has led to such conflicting conclusions that at the present time no definite statement can be made which represents a consensus of opinion as to the function or functions of the lymphocyte.

Bergel,[1, 2] from cultural studies, advanced the theory that lymphocytes possess a lipolytic ferment. However, Reed,[3] Caro[4, 5] and Wiens (as quoted by MacCallum[6]) take exception to the much accepted "Spezifische Fettwerdauende, Phagocytierende Tätigkeit der Lymphocyten" as found by Bergel.

*From the Department of Pathology, University of Oklahoma Medical School.
This study was made possible by Legislative Research grant.
Received for publication, July 5, 1939.

The possible possession of a lipolytic ferment has led to many speculations concerning the role played by the lymphocyte in health and disease. Schaeffer[7] reported that lymphocytes take up fat in the villi and transport it to the lacteals. Many others since Schaeffer have held that the lymphocyte is related to fat metabolism. The statement of Schaeffer seems rather speculative and unproved, and what the relationship, if any, of the lymphocyte to fat metabolism may be is not as yet definitely determined.

Naegeli[8] contends on theoretical grounds that if, as Bergel has stated, the lymphocytes have a special function in secreting a lipoid ferment "a lymphocytosis should be found in all diseases in which the organism exciting the disease contains a lipoid." There would seem to be some confirmation of this theory in the work of Fukushima[9] and Takahashi,[10] who showed that the lipoid isolated from pertussis brought about a lymphocytosis when injected into experimental animals. Opie,[11] however, believes that the lymphocyte contains a protein-digesting ferment which is present only in an acid medium.

Because of the scanty cytoplasm which the cell possesses it has long been thought that lymphocytes are incapable of phagocytosis and for the same reason that they have little power of secretion. Hertzog[12] recently reported that lymphocytes are able to phagocytize. This is as yet unconfirmed and has not been reported under natural conditions, so far as we were able to find.

Murphy and his associates[13-20] undertook extensive investigations into the relation of lymphocytes to resistance in regard to neoplasms and tuberculosis. Their results seem to prove quite definitely that resistance to both transplanted cancer cells and tuberculosis is due to the lymphocytes. It is felt that more critical work should be done in this regard, especially in view of the findings of Loeb[21] in regard to transplant studies. Many other investigations concerning the role of the lymphocyte in relation to tuberculosis and to other diseases, such as whooping cough, seem to bear out the conclusions of Murphy and his associates with respect to other conditions. But none of these authors have given any indication as to the mechanism of this resistance factor.

Because fibroblasts in tissue cultures do better when lymphocytes are added, Carrel[22] believes that the lymphocyte elaborates a growth-promoting substance. Peller[23] also reports that lymphatic tissue is related to growth. But this phenomenon can be better explained on a basis more in harmony with the activities of lymphocytes in other circumstances.

It has been suggested by Bunting and Huston[24, 25] and stated by Maximow,[26] that there is a possibility that lymphocytes bring about a detoxification of bacteria by affixing or absorbing bacterial toxins. Further, Bunting[27] has advanced the hypothesis: "Some mother cell in the lymphoid series produces antitoxins which are then transmitted to her lymphocytic progeny." According to this hypothesis, the lymphocyte would be a passive agent carrying a charge of antitoxic substance. Moor and Newport[28] have experimental evidence that the lymphocyte does have a deleterious effect on pneumococcic toxins. They, as others previously mentioned, feel that this is a phagocytosis phenomenon or an affixing of toxins.

The foregoing citations from the literature prove the chaotic character of our knowledge of the function or functions of the lymphocytes and the need for exact studies of this subject.

Our studies were undertaken (a) to ascertain whether or not lymphocytes do have any effect on the virulence of pneumococci, and (b) if so, to ascertain if possible some indication as to the mechanism of this action.

MATERIALS AND METHODS

The chief difficulty in these studies was to secure a sufficient number of lymphocytes. For this purpose the tonsils were selected as a source of lymphocytes because of the ease of securing this material and the great number of lymphocytes in these structures.

Our method for securing lymphocytes from tonsils is similar to that used by Pappenheimer.[29] Tonsils were obtained immediately following removal. The preparation of the suspension took place as quickly as possible in order to avoid any immediate postmortem changes. The tonsils were washed several times in changes of Ringer's solution, and those showing abscesses and other evidences of infection were discarded. Following the washing process, the periphery of the tonsil was seared with the flat side of a heated scalpel, and hot dissecting needles were plunged into the pits. This procedure was carried out for the purpose of destroying as far as possible surface bacteria. The value of this technique was shown by a noticeable decrease in the number of bacteria in the resulting suspension over that of untreated material.

After surface searing the tonsils were again washed in salt solution and finally placed in 10 c.c. of sterile Ringer's solution in an evaporating dish. The tissue was then teased with needles and gently swirled about in the saline during the operation until the resulting solution was a milky white. The suspension was centrifuged to remove tissue fragments. The cells were then washed twice in sterile Ringer's solution.

Part of the suspension of lymphocytes was incubated for twenty-four hours at 37.5° C., then filtered through a sterile Berkefeld candle. This procedure was carried out in order to secure any extracellular metabolite released by the lymphocytes. Thus, two solutions were secured: (1) a suspension of lymphocytes, and (2) a suspension filtrate. Both these solutions were used in the experiments.

An enumeration of the lymphocytes found in the suspensions was done. It was found that they varied in number from 3,500 to 15,000 per c.mm.

Supravital preparations, using Janus green B, neutral red, and brilliant cresyl blue, were examined on a warm stage. The purpose of this procedure was to determine the numbers of living and dead cells. The method and criteria were those used by Pappenheimer.[29] Movement of mitochondria about the nucleus in a semicircular formation was noted. Centrioles were observed dancing about in the cytoplasm and were identified as being quite possibly the bodies described by Gall[30] in the cytoplasm of the living lymphocyte. According to these criteria, lymphocytes may be found alive in these suspensions on the third day of incubation and motile macrophages have been found on the fifth day.

Variations in the size of the lymphocytes were noted, although they appeared to be essentially of the small variety. A few macrophages were observed but they are apparently fewer than expected. The lymphocytes were not observed phagocytizing at any time, even though it has been recently claimed that they may phagocytize under certain conditions (Hertzog[31]). Cells were observed which contained large vacuoles, approaching in some instances the size of the nucleus. Small cytoplasmic bodies, similar to those described by Downey and Weidenreich,[32] were seen budding off from the living cells and were observed in dry preparations.

A gradual formation of a white flocculent precipitate of the suspension was noted. The precipitation began immediately and continued until a clear supernatant liquor resulted. Upon microscopic examination it was seen that these white, flecklike particles were agglomerations of bacteria about lymphocytes which formed the center or nucleus of such groups. The centrioles of the lymphocytes in most cases were very active; hence the cells were alive. The bacteria in a group adhere to each other and thus form larger masses of organisms. It is interesting to note the agglomeration included all forms of bacteria and even spirilla, and that motile bacteria held in the agglomerations were observed attempting to gain a release from the mass. This agglomeration, therefore, was not specific for any type or species of organism.

Whether or not lymphocytes take part in the formation of agglutinins requires further investigation. There is some evidence that lymphocytes may be associated with the production of agglutinins (McMaster and Hudack,[33] Simonds and Jones[35]). Lowen found a decrease of specific agglutinins during experimental lymphopenia. Williams and Dougherty[36] found an increase in complement following aleuronat injections in the guinea pig. A peripheral lymphocytosis appears to occur at certain stages following the injections of aleuronat. In clinical laboratory procedure most diseases in which serum reactions have the greatest diagnostic value are characterized by a lymphocytosis or lymphadenitis. Therefore, in the observations in regard to agglutinins previously noted, the phenomena may be due to a missing link in the chain, rather than to positive agglutinogenesis.

In our studies no evidence of agglutinins was found. The phenomenon described was an agglomeration and required the actual presence of the lymphocyte for the phenomenon to occur.

EXPERIMENTS

In order to test the activity of the lymphocyte in a more or less natural state the sputum from pneumonic patients was diluted with sterile normal saline and shaken vigorously to secure a uniform distribution of bacteria. The pneumococci were typed and their virulence ascertained previous to using the sputum in the experiments. The amount inoculated varied from 0.1 c.c. to 0.5 c.c. The total dose of the sputum, regardless of dilution, was made up to 1 c.c., which was the total dosage in all cases. Inoculations were made with a 1 c.c. tuberculin syringe and a short needle.

White mice were used in the experiments because they are extremely susceptible to small doses of virulent pneumococci and because they are so

widely used in the laboratory to test the virulence of the pneumococci in sputum samples.

Control animals were injected with varying amounts of sputum. The experimental animals were injected with sputum which had been added to the lymphocyte suspensions and filtrates. Two sets of experiments were carried out. In one the mixture of sputum and lymphocytes was injected immediately upon being made up. In the other the mixture was allowed to remain in the incubator at 37.5° C. for varying periods of time previous to injection. The reason for this variation was to ascertain whether or not a time relationship at body temperature might affect the results.

The survival time of the animals was taken as a criterion of the detoxification effect of the lymphocytes. The animals were carefully observed constantly during the day and at three-hour intervals during the night. If an animal was found dead at the end of a three-hour period, the former period was taken as the survival time of the animal. Autopsies were made on all animals in an attempt to ascertain as far as possible whether or not death might have been due to a puncture of the intestine. Each animal was marked with dye, and each group of animals was kept separate during the course of the experiment. All pertinent data were kept as to identification, time of inoculation and death, amount injected, and treatment to which the mixture had been subjected.

TABLE I

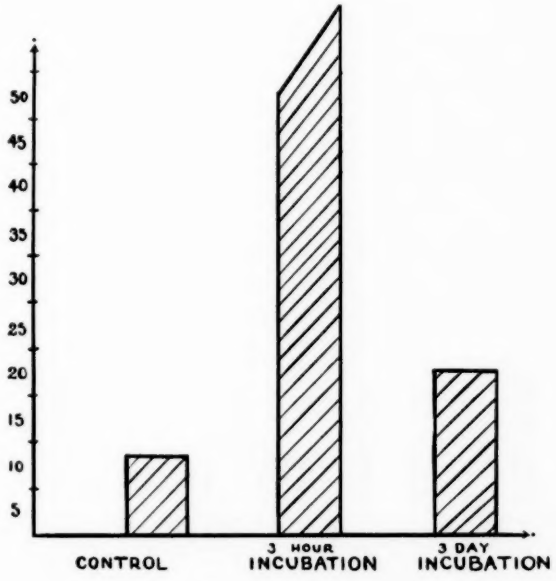
TYPE VII PNEUMOCOCCUS

ANIMAL NO.	TYPE	AMOUNT INOCULATED	INCUBATION	SURVIVAL TIME
1	Control	0.5 c.c. suspension	----	----
2	Control	0.5 c.c. suspension	----	----
3	Control	0.5 c.c. sputum 0.5 c.c. saline	----	7 hours
4	Control	0.5 c.c. sputum 0.5 c.c. saline	----	7.5 hours
5	Experiment	0.5 c.c. sputum 0.5 c.c. suspension	----	48 hours
6	Experiment	0.5 c.c. sputum 0.5 c.c. suspension	3 hours	48 hours
7	Experiment	0.5 c.c. sputum 0.5 c.c. suspension	3 hours	48 hours
8	Experiment	0.5 c.c. sputum 0.5 c.c. suspension	72 hours	18 hours
9	Experiment	0.5 c.c. sputum 0.5 c.c. suspension	72 hours	16 hours

Experiment I.—Type VII pneumococcus was used in the first investigation. The mice, approximately 20 Gm. in weight, were all kept separate during the course of the experiment. The amount of sputum-lymphocyte mixture is noted in Table I. The filtrate was not used in this series. The incubatory period of the sputum and the lymphocyte mixture varied from zero to seventy-two hours. The amount of sputum was kept the same in all injections. Two controls injected with lymphocytes alone showed no untoward symptoms, such as an anaphylactoid response.

There is quite a notable difference in the survival time of the control and the experimental animals (Table I and Graph 1). The three mice which

survived the interval of forty-eight hours were fully as sick as the control mice for the first twenty-four hours, after which time they began to improve, and about the fourth day were quite as normal in appearance and appetite as before the experiment. Mice 8 and 9 which received the sputum and lymphocyte mixture, which had been incubated for seventy-two hours, lived only seventeen hours (about 2.5 times as long as the control). It will be noted that the animals surviving the forty-eight-hour interval lived approximately 6.5 times as long as the control. The average for the group based on the forty-eight-hour interval was thirty-four hours. From the above it seems evident that the lymphocyte had no deleterious effect on the bacteria and that the survival time in these groups depended on the proportion of living lymphocytes present.



Graph 1.—Type VII pneumococcus.

Experiment II.—Sputum containing type III pneumococcus was used in the second set of experiments. The procedure followed was essentially that which was used in the experiments with type VII strain, with the exception that the bacteria were given treatment with filtrate as well as with the tonsillar suspension.

The amount of sputum used in the various experiments is noted in Tables II, III, and IV, and the average survival time of the animals in each group is noted on Graph 2.

The sputum was injected in varying amounts: 0.1 c.c., 0.25 c.c., and 0.5 c.c. In each instance filtrate or suspension was added to the sputum and was placed in the incubator for a twenty-four-hour period. The survival time of the animals was carefully noted. Control animals were injected with the same amount of sputum as the test animals, with the exception that physiologic salt solution was added to make the total dose 1 c.c.

TABLE II

ANIMAL NO.	TYPE	AMOUNT INOCULATED	INCUBATION	SURVIVAL TIME
1	Control	0.5 c.c. sputum 0.5 c.c. saline	24 hours	7.5 hours
2	Control	0.5 c.c. sputum 0.5 c.c. saline	24 hours	8 hours
3	Experiment	0.5 c.c. sputum 0.5 c.c. filtrate	24 hours	12 hours
4	Experiment	0.5 c.c. sputum 0.5 c.c. filtrate	24 hours	6 hours
5	Experiment	0.5 c.c. sputum 0.5 c.c. filtrate	24 hours	3 hours
6	Experiment	0.5 c.c. sputum 0.5 c.c. filtrate	24 hours	3 hours
7	Experiment	0.5 c.c. sputum 0.5 c.c. filtrate	24 hours	12 hours
8	Experiment	0.5 c.c. sputum 0.5 c.c. suspension	24 hours	17 hours
9	Experiment	0.5 c.c. sputum 0.5 c.c. suspension	24 hours	17 hours

TABLE III

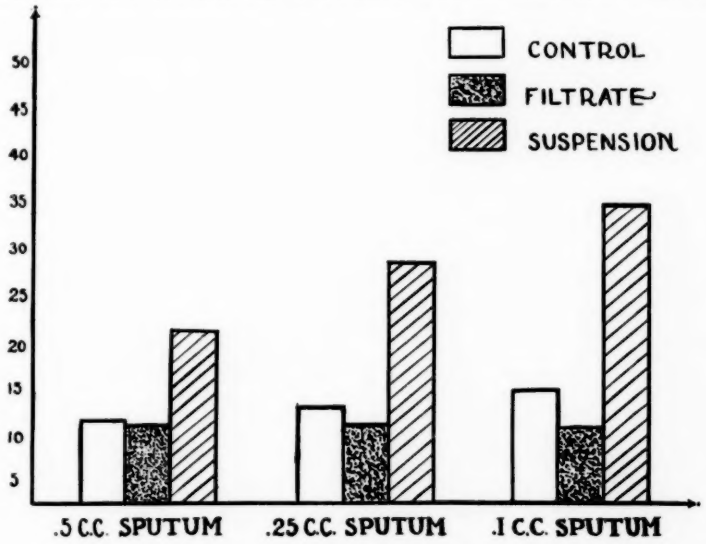
ANIMAL NO.	TYPE	AMOUNT INOCULATED	INCUBATION	SURVIVAL TIME
1	Control	0.25 c.c. sputum 0.25 c.c. saline	24 hours	9 hours
2	Experiment	0.25 c.c. sputum 0.75 c.c. filtrate	24 hours	9 hours
3	Experiment	0.25 c.c. sputum 0.75 c.c. filtrate	24 hours	6.5 hours
4	Experiment	0.25 c.c. sputum 0.75 c.c. suspension	24 hours	6 hours
5	Experiment	0.25 c.c. sputum 0.75 c.c. suspension	24 hours	15 hours
6	Experiment	0.25 c.c. sputum 0.75 c.c. suspension	24 hours	21 hours
7	Experiment	0.25 c.c. sputum 0.75 c.c. suspension	24 hours	33 hours
8	Experiment	0.25 c.c. sputum 0.75 c.c. suspension	24 hours	46 hours

TABLE IV

ANIMAL NO.	TYPE	AMOUNT INOCULATED	INCUBATION	SURVIVAL TIME
1	Control	0.1 c.c. sputum 0.9 c.c. saline	24 hours	9 hours
2	Control	0.1 c.c. sputum 0.9 c.c. saline	24 hours	14 hours
3	Experiment	0.1 c.c. sputum 0.9 c.c. filtrate	24 hours	6.5 hours
4	Experiment	0.1 c.c. sputum 0.9 c.c. filtrate	24 hours	8 hours
5	Experiment	0.1 c.c. sputum 0.9 c.c. suspension	24 hours	30 hours
6	Experiment	0.1 c.c. sputum 0.9 c.c. suspension	24 hours	36 hours
7	Experiment	0.1 c.c. sputum 0.1 c.c. suspension	24 hours	27 hours

The individual or average survival time did not deviate materially according to the amount of sputum injected. Hence it may be assumed that the lethal dose was somewhat lower. The averages presented in Graph 2 are based upon the varying amount of sputum injected, although the average survival time in each of the groups is very similar.

The average survival time of the control animals injected with 0.5 c.c. of sputum was 7.5 hours; the filtrate animals averaged 7.3 hours and the suspension animals 17 hours. The control animals receiving 0.25 c.c. of sputum lived 9 hours; the filtrate group 7.3 hours and the suspension group 24.6 hours. The control animals receiving 0.1 c.c. of sputum lived 9 hours; the filtrate group 7.25 hours and the suspension group 26.7 hours. If the survival time of all of the animals in each group are averaged together, it may be observed that the control survival time was 8.5 hours, the filtrate group



Graph 2.—Type III pneumococcus.

7.5 hours, and the suspension group 22.7 hours. Thus, the survival time of the individual groups closely approximates that of the whole. It is also observed that the animals receiving the filtrate of the lymphocyte suspension did not live longer than the control. The small variation on this number of animals is not significant. The animals receiving the suspension lived approximately three times as long as either of the other groups.

This experiment, using both the filtrate and the suspension, was done to ascertain whether the lymphocytes produced some extracellular detoxifying substance as well as to determine whether or not lymphocytes played any part as a defense mechanism in pneumococcus infection.

From the foregoing data it seems to be evident (1) that lymphocytes do not produce any extracellular substance deleterious to pneumococci type III; (2) that living lymphocytes when present do have a detoxifying power on pneumococci type III; and (3) that the theory of an inheritance by the lymphocyte of a detoxifying substance from "some mother cell," as suggested by Bunting, is untenable.

CONCLUSIONS

A careful analysis of our experiments seems to justify the following conclusions:

1. Lymphocytes play a part in the resistance of white mice to the action of pneumococci types III and VII.

2. There was no evidence in our experiments that lymphocytes produce any extracellular metabolite which plays any part in the phenomena noted.

3. No prolongation of survival time occurred in any of our experiments, except when the living lymphocytes were present.

4. The detoxifying action of lymphocytes on pneumococci in our experiments was in inverse proportion to the virulence of the type.

5. Since the exposure of the pneumococci to lymphocytic suspension filtrate, and since a seventy-two-hour incubation with lymphocytes did not proportionally prolong the survival time of the animals (Graph 1), it would seem that the protective effect of lymphocytes on animals to pneumococci was not due to metabolites or products of autolysis of the cells, as suggested by Moor and Newport,[28] as a possible mechanism.

6. A consideration of these data would seem to indicate that the protective action of lymphocytes to pneumococci was a quantitative one, depending upon the viability of the lymphocytes, and that the mechanism was one of ingestion and intracellular disposition.

REFERENCES

1. Bergel, S.: Beitrage zur Biologie der Lymphozyten, Ztschr. f. exper. Path. u. Therap. **21**: 216, 1920.
2. Bergel, S.: Weiteres zur Lipoidspaltenden Funktion der Lymphozyten, Beitr. z. path. Anat. u. z. allg. Path. **73**: 404, 1925.
3. Reed, C. C.: Studies on Biochemistry and Chemotherapy of Tuberculosis, Fat-Splitting Ferments in Lymphocytes, Am. Rev. Tubere. **7**: 105, 1923.
4. Caro, L.: Fettspaltende ferment im menschlichen Blut serum, ihre Abhangigkeit von krankhaften, namentlich kachektischen Zustanden, ihre Unabhangigkeit von der histologischen zusammensetzung des Blutes, Ztschr. f. klin. Med. **78**: 286, 1913.
5. Caro, L.: Zur frage der Herkunft und Bedeutung von fettspaltenden Fermenten des menschlichen Blutes, Ztschr. f. klin. Med. **89**: 49, 1920.
6. MacCallum, W. G.: A Textbook of Pathology, Philadelphia, 1916, W. B. Saunders Co., p. 150.
7. Schaeffer, E. A.: On the Part Played by Amoeboid Cells in the Process of Intestinal Absorption, Internat. Monatschr. f. Anat. u. Physiol. **2**: 6, 1885.
8. Naegeli, Otto: Blutkrankheiten und Blutdiagnostik, ed. 5, Berlin, 1931, Julius Springer, p. 134.
9. Fukushima, M.: Chemical Analysis of Bacilli of Whooping Cough, Orient. J. Dis. Infants **3**: 14, 1928.
10. Takahashi, S.: Biologische und Serologische Studien über Bordetsche Keuchhustenbazillen, Sei-I-Kai M. J. **49**: 4, 1930.
11. Opie, Eugene L.: Intra Cellular Digestion: The Enzymes and Antienzymes Concerned, Physiol. Rev. **2**: 552, 1922.
12. Hertzog, A. J.: The Phagocytic Activity of Human Leukocytes With Special Reference to Their Type and Maturity, Am. J. Path. **14**: 595, 1938.
13. Murphy, James B.: Studies in Tissue Specificity. III. Factors of Resistance to Heteroplastic Tissue Transplants, J. Exper. Med. **19**: 531, 1914.
14. Idem: Studies in Tissue Specificity. II. Ultimate Fate of Mammalian Tissue Implanted in the Chick Embryo, J. Exper. Med. **19**: 181, 1914.
15. Murphy, J. B., and Ellis, A.: Experiments on the Role of Lymphoid Tissue in the Resistance to Experimental Tuberculosis in Mice, J. Exper. Med. **20**: 397, 1914.
16. Murphy, J. B., and Morton, John J.: Studies in Lymphoid Activity. II. The Lymphocyte in Natural and Induced Resistance to Transplanted Cancer, J. Exper. Med. **22**: 204, 1915.

17. Murphy, J. B., and Morton, J. J.: The Effect of Roentgen Rays on the Rate of Growth of Spontaneous Tumors in Mice, J. Exper. Med. **22**: 801, 1915.
18. Murphy, J. B., and Sturm, E.: The Effect of Dry Heat on the Blood Count of Animals, J. Exper. Med. **29**: 1, 1919.
19. Murphy, J. B., and Nakahara, W.: The Lymphocytes in Natural and Induced Resistance to Transplanted Cancer. V. Histological Study of the Lymphoid Tissue of Mice With Induced Immunity to Transplanted Cancer, J. Exper. Med. **31**: 1, 1920.
20. Murphy, J. B., Nakahara, Waro, and Sturm, E.: Studies on Lymphoid Activity: Relation Between the Time and Extent of Lymphoid Stimulation Induced by Physical Agents and the Degree of Resistance to Cancer in Mice, J. Exper. Med. **33**: 423, 1921.
21. Loeb, Leo: The Individuality Differential and the Reactions Against Transplanted Tissues and Tumors With Particular Reference to Lymphocytic Reaction, J. Cancer Research **10**: 252, 1926.
22. Carrel, A.: Leukocytic Trephones, J. A. M. A. **82**: 255, 1924.
23. Peller, S.: Ueber die Funktion der Tonsillen, Ztschr. f. menschl. Vererb.-Konstitutionslehre **17**: 604, 1933.
24. Bunting, C. H., and Huston, J. H.: Fate of the Lymphocyte, J. Exper. Med. **33**: 593, 1921.
25. Bunting, C. H.: Handbook of Hematology, New York, 1938, Paul B. Hoeber, Inc., p. 442.
26. Maximow, Alexander A., and Bloom, W.: Textbook of Histology, Philadelphia, 1931, W. B. Saunders Co., p. 496.
27. Bunting, C. H.: Handbook of Hematology, New York, 1938, Paul B. Hoeber, Inc., p. 443.
28. Moor, H. D., and Newport, N. M.: The Effect of Lymphocytes in Vitro Upon Bacterial Toxins, J. Lab. & Clin. Med. **24**: 471, 1939.
29. Pappenheimer, A. M.: Experimental Studies on Lymphocytes. I. The Reaction of Lymphocytes Under Various Experimental Conditions, J. Exper. Med. **25**: 633, 1917.
30. Gall, E. A.: A Previously Undescribed Granule Within the Lymphocyte, Am. J. M. Sc. **191**: 380, 1936.
31. Hertzog, A. J.: Ibid.
32. Downey, Hal, and Weidenreich, F.: Ueber die Bildung der Lymphozyten in Lymphdrusen und Milz. IX. Fortsetzung der "Studien ueber das Blut und die Blut Bildenden und zerstoerenden Organe," Arch. f. Mikr. Anat. **80**: 306, 1912.
33. Masiaster, P. D., and Hudack, S. S.: The Formation of Agglutinins Within Lymph Nodes, J. Exper. Med. **61**: 783, 1935.
34. Simonds, J. P., and Jones, H. M.: The Influence of the Exposure to X-rays Upon the Formation of Antibodies, J. M. Research **83**: 183, 1915.
35. Simonds, J. P., and Jones, H. M.: The Effect of Injections of Benzol Upon the Production of Antibodies, J. M. Research **33**: 197, 1915.
36. Williams, O. O., and Dougherty, Tom: Effects of Aleuronat Injection on Complementing Activity of Guinea Pig Serum, J. Immunol. **36**: 559, 1939.

PROTECTION AGAINST RABIES*

I. The Effect of Frequency of Dosage of Vaccine Upon Immunity

James M. Moss, M.D., Indianapolis, Ind.

A RECENT article by Blatt and co-workers[1] in which twelve deaths from rabies were reported has again impressed upon all the necessity of early adequate immunization as well as sufficient epidemiologic precautions in rabies exposure.

There still exists some controversy as to the best method of acquiring immunity to rabies, especially in reference to the frequency of administration of vaccine. The lack of a satisfactory biological test for rabies immunity has in the main been responsible for the lack of information as to the antigenic response obtained in animals. In recent years certain strains of mice have been found by Webster and Dawson[2] to be susceptible to intracerebral injection of rabies virus. This has offered a method of titrating antivirus serum against rabies virus.

We have been interested in determining the antigenic response to a variable number of daily doses of rabies vaccine. Many clinicians feel that the administration of two injections of rabies vaccine daily gives rise to a more rapid immunity. It has been our impression that the reverse might be accomplished by the too frequent administration of antigen.

EXPERIMENTAL

In order to determine the comparative immunity response to varying frequency of doses, 7 groups of rabbits received a variable number of daily injections of rabies vaccine. Each of the 7 groups consisted of 9 rabbits. The first 5 groups I to V received vaccine made by the Harris method; while groups VI and VII received vaccine made by the Semple process. The dosage used was the same as the amount of vaccine administered in human immunization, namely 0.5 c.c. of Harris vaccine and 2.0 c.c. of Semple vaccine. Both vaccines were prepared and used in a fresh state before antigenic potency had an opportunity to deteriorate.

Each of the Harris groups received a total of fourteen subcutaneous injections. Group VI received fourteen inoculations of Semple vaccine, while group VII received a total of twenty-one injections of the Semple vaccine.

Chart 1 gives the schematic plan of injection for each group and also records the deaths from rabies encephalitis. It is interesting to note that in this series the number of deaths from encephalitis is roughly in ratio to the frequency of injections.

*Eli Lilly Research Fellowship, Indiana University School of Medicine, Indianapolis.
Received for publication, July 10, 1939.

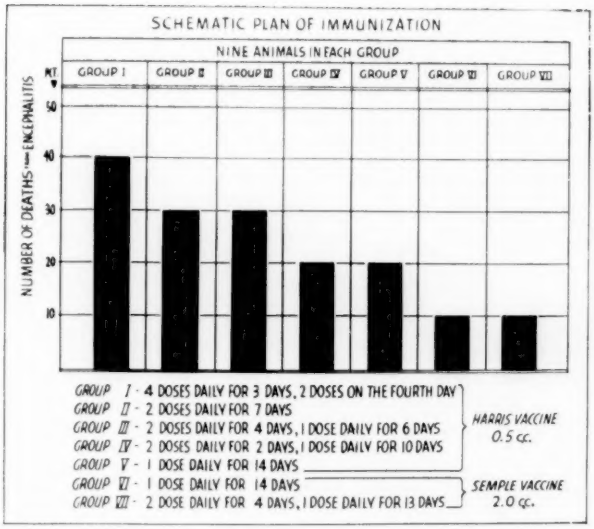

SCHEMATIC PLAN OF IMMUNIZATION
NINE ANIMALS IN EACH GROUP
GROUP I
GROUP II
GROUP III
GROUP IV
GROUP V
GROUP VI
GROUP VII
NUMBER OF DEATHS — ENCEPHALITIS
GROUP I - 4 DOSES DAILY FOR 3 DAYS, 2 DOSES ON THE FOURTH DAY
GROUP II - 2 DOSES DAILY FOR 7 DAYS
GROUP III - 2 DOSES DAILY FOR 4 DAYS, 1 DOSE DAILY FOR 6 DAYS
GROUP IV - 2 DOSES DAILY FOR 2 DAYS, 1 DOSE DAILY FOR 10 DAYS
GROUP V - 1 DOSE DAILY FOR 14 DAYS
GROUP VI - 1 DOSE DAILY FOR 14 DAYS
GROUP VII - 2 DOSE DAILY FOR 4 DAYS, 1 DOSE DAILY FOR 13 DAYS
HARRIS VACCINE 0.5 cc.
SEMPLE VACCINE 2.0 cc.

Chart 1.

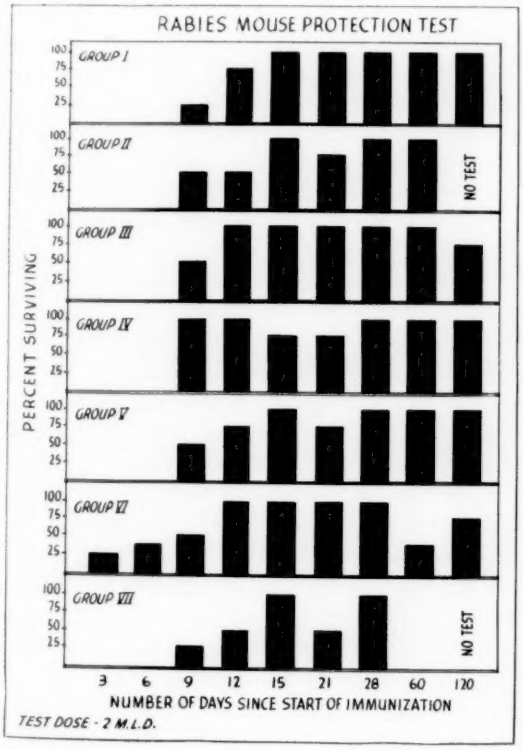


Chart 2.

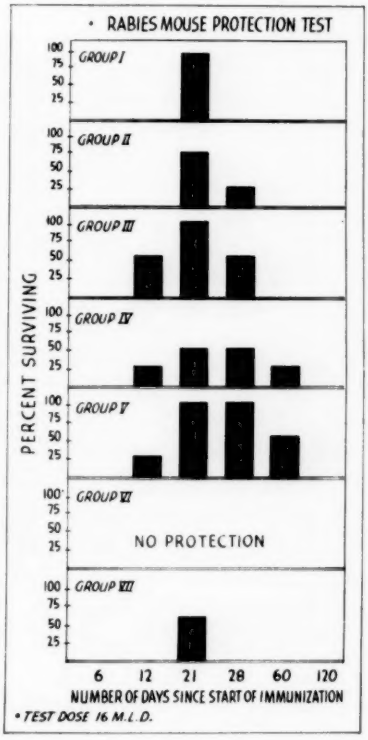


Chart 3.

IMMUNITY TESTS

Bleedings were made from each group of animals on the third, sixth, ninth, twelfth, fifteenth, twenty-first, twenty-eighth, sixtieth, and one hundred and twentieth days after the first injection of vaccine, with the exception of groups II and VII at 120 days. The sera of the individual groups were pooled to limit variation of animal response.

Each specimen of serum from the respective groups was titrated against two minimal lethal doses of emulsified rabies virus. Two hundredths cubic centimeter (0.02) serum from each bleeding was mixed with 0.02 c.c. of a 1:99 dilution with emulsified virus, which is equal to two minimal lethal doses. The mixture was allowed to incubate at 37° C. in a water bath for one hour. The virus-serum mixture was then injected intracerebrally into four white mice. All series were controlled with varying dilutions of rabies virus mixed with normal rabbit serum. All mice that succumbed did so around the ninth or tenth day. Surviving mice were observed until the twentieth day.

Chart 2 illustrates the protection of the serum from each group when titrated against a test dose of two minimal lethal doses of rabies virus. All controls died by the ninth day. In this chart the percentage of immunity is determined by the percentage of mouse survival. Only group VI (Semple vaccine) demonstrates immunity at the third and sixth days. All groups show some protection at the ninth day.

Groups I and II, which received multiple daily doses of vaccine throughout, did not respond as rapidly as groups III, IV, V, and VI up to the fifteenth day of immunization. It is at that point that sufficient response in all the groups is obtained to protect against two minimal lethal doses almost completely. Group VII has the poorest early response.

Serum-virus neutralization begins to decrease in groups VI and VII as early as the sixtieth day; group VI shows only 33⅓ per cent demonstrable protection, and group VII no protection. In the Harris groups all animals survived at this point. At four months all Harris groups except group III protect 100 per cent, while group VI protects 75 per cent. No tests were made on groups II and VII at four months.

Using only two minimal lethal doses in high dilutions injected intracranially, a small percentage of error may be expected, even though we have a 100 per cent mortality in our controls.

In order to gain a better comparison of the total immunity obtained, the bleedings from all groups were titrated against an infective dose of sixteen minimal lethal doses. The results are shown in Chart 3. In these titrations bleedings on the third, ninth, and fifteenth days were omitted.

Titrating each specimen of serum against sixteen minimal lethal doses we find some protection present as early as the twelfth day in groups III, IV, and V. All of the Harris groups show some protection on the twenty-first day. On the twenty-eighth day all groups except I and VI show some protection. Only groups IV and V show some protection at two months. Protection is not demonstrable in group VI at any time, and there is only 50 per cent protection in group VII on the twenty-eighth day.

It is obvious from Chart 3 that doubling doses with the Harris vaccine decreases the antigenic response, diminishes the rapidity of rise of antibody formation, and shortens the duration of immunity. The attenuated Harris vaccine has given a higher measurable amount of immunity than the killed Semple vaccine, with a longer duration of immunity.

Group V in Table III would seem to demonstrate that best results are obtained from single daily injections of vaccine.

DISCUSSION

Many clinicians feel that it is advisable to give multiple daily injections of rabies vaccine in an attempt to stimulate a more rapid response of antibody formation. This is especially true in cases of bites about the head or in close proximity to the central nervous system. The procedure is sometimes used when the starting of treatment has been delayed for various reasons, such as the reluctance of a patient to take treatments or the delayed diagnosis of the biting animal.

It has been our conception that immune response to rabies vaccine would probably resemble that obtained from the administration of certain bacterial antigens. In the production of either tetanus or diphtheria antitoxin small doses of antigen administered at longer time intervals give a higher titer than large doses injected at more frequent intervals. In fact, immune response with those antigens seems to be retarded by large frequent injections. That the same fact is true with rabies vaccine is indicated by the experiments reported here. In Chart 2, with the immune serum titrated against an infective dose of two minimal lethal doses of virus, all groups show a fair percentage of immunity as early as the ninth and twelfth days. Most of the groups are more or less uniform in their response at different intervals of titration.

In order to determine the presence of wide variation in response, tests were run against a much larger infective dose, namely, sixteen minimal lethal doses. Under these conditions real differences are noted, and as shown by Chart 3, there seems to be some ratio of a more pronounced early immune response with a longer duration of immunity, to decreasing the frequency of daily doses.

The immune response in Chart 3 is greater in the groups that received vaccine made by the Harris method than in the groups that received vaccine prepared by the Semple method. This is in accord with Webster's[3] observations in which he states that "vaccines in which virulence has been reduced by phenol are correspondingly less effective as immunizing agents."

There is no information at present concerning the amount of antibody formation needed to protect against rabies. Yet it seems the method of choice in immunization would be that which gives the highest early response with the longest duration of immunity. In our series the groups fulfilling these requirements received but one injection of vaccine daily prepared by the Harris method.

CONCLUSIONS

1. Administration of multiple daily doses of rabies vaccine seems to retard immune response.

2. In our experiments an earlier response with a longer duration of immunity was obtained by single daily doses of vaccine made by the Harris method.

I wish to acknowledge my thanks to Mr. W. A. Jamieson for his generous advice and assistance throughout this investigation.

REFERENCES

1. Blatt, M. L., Hoffman, S. J., and Schneider, M.: Rabies. Report of Twelve Cases, With Discussion of Prophylaxis, J. A. M. A. **111**: 688, 1938.
2. Webster, L. T., and Dawson, J. R., Jr.: Early Diagnosis of Rabies by Mouse Inoculation. Measurement of Humoral Immunity to Rabies by Mouse Protection Test, Proc. Soc. Exper. Biol. & Med. **32**: 570, 1935.
3. Webster, L. T.: Epidemiologic and Immunologic Experiments on Rabies, New England J. Med. **217**: 687, 1937.

TRANSFUSION OF BLOOD FROM ARTIFICIALLY IMMUNIZED DONOR IN THE TREATMENT OF CHRONIC BACILLARY DYSENTERY*

ROBERT TURELL, M.D., NEW YORK, N. Y.

THE purpose of this communication is to record the observations with transfusion of blood obtained from artificially immunized donors and used in the treatment of chronic bacillary dysentery. Artificial immunization was produced by the injection of a total of 5 c.c. of the recipient's autogenous vaccine into a suitable donor, without apparent ill effects. An initial dose of 0.1 c.c. of vaccine was given intracutaneously. Three days later, 0.1 c.c. was injected intracutaneously and 0.1 c.c. subcutaneously. Subsequently, the intracutaneous dose remained unchanged, but the subcutaneous one was increased by 0.1 c.c. until 0.5 c.c. was given at one injection.

CASE REPORT

Mrs. B. G., 28 years old, was seen in the office on May 6, 1938, because of bloody diarrhea and loss of weight. In February, 1930, immediately after an appendectomy, she passed bright blood in the stool. Two years later she had an acute attack of diarrhea of three weeks' duration. Bloody stools were preceded by generalized abdominal pain. During the spring of the years 1933, 1934, and 1935, she had mild attacks of diarrhea, each episode lasting three months. In 1935 she lost 50 pounds. During the subsequent three years the diarrhea was persistent, and she had 6 to 8 bowel movements daily.

The family history revealed that her brother died of colitis of undetermined origin, and that her mother has had diarrhea for seventeen years. Her blood serum agglutinated Flexner antigens in dilution of 1:320.[1]

The pertinent points in the physical examination were pallor, evidence of loss of weight, and a scaphoid abdomen, with tenderness on moderate pressure in both lower quadrants. The sigmoidoscopic examination revealed a finely granular and easily bleeding mucosa. At the rectosigmoid, the mucous membrane was swollen, thickened, and covered with deep ulcers—a picture of a severe ulcerative colitis.

The bacteriologic studies of the material obtained from these ulcers yielded an atypical Flexner dysentery organism, from which an autogenous vaccine was prepared by Eli Lilly and Company. The stool showed numerous leucocytes and erythrocytes.

*From the Department of Surgery, Division of Proctology, the Brooklyn Hospital, Brooklyn. Dr. A. W. Martin Marino, attending proctologist.

Read before the New York Proctologic Society, New York City, April 20, 1939.
Received for publication, July 12, 1939.

Her blood serum agglutinated Flexner antigens in dilutions of 1:640 on nine different occasions. The blood count revealed hemoglobin 50 per cent (Sahli), 4,770,000 erythrocytes, 10,300 leucocytes, of which segmented polymorphonuclear cells constituted 62 per cent; staff cells 2 per cent; lymphocytes 29 per cent; eosinophiles 5 per cent, and monocytes 2 per cent.

The roentgen-ray examination of the colon, with the aid of a barium enema, showed an ulcerative colitis involving the entire large bowel. There was loss of haustration, narrowing of the lumen, and absence of the mucosal pattern without irritability. The terminal ileum appeared normal.

The patient was not seen again until Sept. 26, 1938. At that time, she had profuse bloody diarrhea, abdominal cramps, and distention, and a fever of 103° F. Two days later she was admitted to the Brooklyn Hospital. Although her general condition improved under supportive treatment and transfusions of blood, the diarrhea continued at a rate of 10 to 15 bowel movements daily. The stools were semiformed or fluid, and mixed with pus, mucus, and blood. Following the administration of autogenous vaccine, the frequency of the diarrhea was reduced to 4 to 6 bowel movements daily.

She was sent home on Nov. 6, 1938. Vaccinotherapy was continued. She showed satisfactory improvement, gained 12 pounds in weight during one month, menstruated for the first time in over six months, and had 4 to 5 bowel movements daily. The stools were semiformed or formed. The temperature ranged from 98.6° to 99° F.

On Jan. 6, 1939, two months after she left the hospital, she developed an upper respiratory infection which was associated with a temperature of 102° F., and sharp pains in the wrist, elbow, and shoulder joints. This episode lasted ten days, and was followed by another one on January 21, and again on February 2. During the last episode the diarrhea increased to 8 to 10 bowel movements daily, and the temperature rose to 102.5° F. in the late afternoon and early evening; she experienced abdominal cramps and intermittent pain in the joints. During this time vaccinotherapy on two occasions appeared ineffective, and was, therefore, suspended temporarily.

On Feb. 14, 1939, the patient was readmitted to the Brooklyn Hospital, and an immunotransfusion of 300 c.c. of whole blood was given four days later. On the day of the transfusion she had 4 bowel movements, and continued to have 4 to 5 daily bowel movements with the exception of one day. The temperature remained normal for over a month, but since then has varied from normal to 99.4° F. Her general condition improved satisfactorily, but the pain in the joints was not affected. The blood count four days prior to immunotransfusion was: hemoglobin 68 per cent (Sahli); erythrocytes 3,310,000; leucocytes 20,600, of which polymorphonuclear cells constituted 81 per cent; and the lymphocytes 19 per cent. Three days after the immunotransfusion, the blood count was hemoglobin 76 per cent; erythrocytes 3,940,000; leucocytes 7,250, of which the polymorphonuclears constituted 62 per cent; lymphocytes 24 per cent; and eosinophiles 14 per cent.

To date she has been having 4 to 5 bowel movements daily. The stools are either semiformed or formed. She is gaining in weight, but a nonproductive cough and pains in the left wrist and right shoulder joints still persist.

COMMENT

The response of this patient to transfusions of blood was studied in the fall of 1938. At that time the donor had given 500 c.c. of plain blood (September 29) and later 300 c.c. (October 23). The beneficial results from those transfusions could in nowise be compared with those noted after the transfusion of immunized blood from the same donor. The effect on the diarrhea was definite and clear-cut. On the third day after the immunotransfusion, there was a drop, perhaps coincidental, in the number of leucocytes from 20,600 to 7,250. The increase in the hemoglobin and the number of erythrocytes was minimal.

My observations on artificially produced immunotransfusion seem to parallel those noted from transfusions of blood obtained from patients who recovered from bacillary dysentery.[2, 3]

SUMMARY

Observations are reported on the effects following a transfusion of 300 c.c. of blood from a donor who was artificially immunized with a vaccine prepared from an atypical Flexner dysentery organism isolated from the ulcers of the recipient's colon.

The practical results obtained in this case and the theoretical basis of this method would appear, in my opinion, to justify an extensive trial of this type of immunotransfusion in order to determine its proper place in therapy.

REFERENCES

1. Turell, R.: The Relationship of Bacillary Dysentery Infections to Chronic Intermittent Diarrheas, Rev. Gastroenterol. **7**: 14, 1940.
2. Felsen, J.: Relationship of Bacillary Dysentery to Distal Ileitis, Chronic Ulcerative Colitis and Non-Specific Intestinal Granuloma, Ann. Int. Med. **10**: 645, 1936.
3. Felsen, J.: Personal communication to the author.

THE EFFECTS OF NICOTINE AND CIGARETTE SMOKE ON PREGNANT FEMALE ALBINO RATS AND THEIR OFFSPRINGS*

J. M. Essenberg, Justin V. Schwind, and Anne R. Patras, Chicago, Ill.

INTRODUCTION

INTEREST in the present study was aroused by one of the questions most commonly asked at the embryologic exhibit of Loyola University at the Chicago World's Fair of 1933-34. The question was: "How does tobacco smoke affect the unborn child?"

Upon review of the literature on the effect of tobacco smoke and nicotine on human beings and experimental animals several limitations were encountered. First, the number of studies done on this problem is relatively small as compared to other problems of equal importance: this is especially true of studies dealing with pregnancy. Second, the findings of various authors are too contradictory to permit the drawing of reliable conclusions. This state of affairs is, at least in part, responsible for the laissez-faire attitude that many physicians take in advising their patients during pregnancy. Third, in many instances the number of animals used in the experiment is too small to gain convincing results. Fourth, far-reaching conclusions have been drawn by some writers from general observation of tobacco users but with no specific study of human beings and animals. This, naturally, has added more confusion to the tobacco problem.

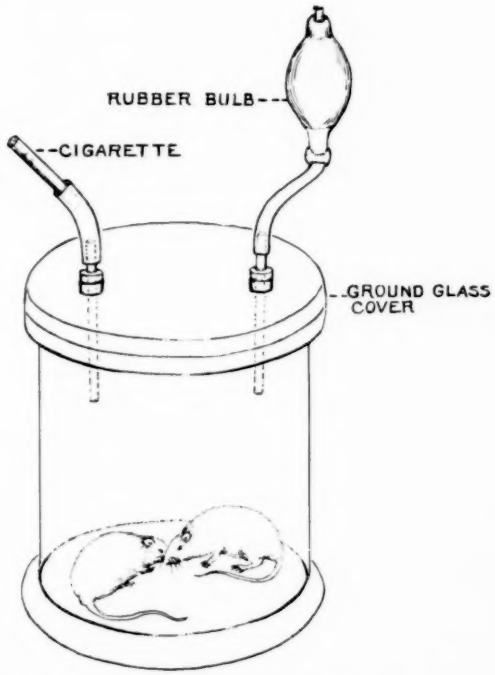
When all of the foregoing facts are considered, the conclusion is that the tobacco problem has not been sufficiently studied. It will require the attention of many investigators before an unbiased answer to the question can be given.

*From the Department of Anatomy, Loyola University, School of Medicine, Chicago.
Received for publication, July 29, 1939.

The question was ripe at the time it was put to us. It is more ripe today and it may prove to be more serious than is generally recognized.

MATERIALS AND METHODS

In the selection of our materials and methods we have constantly tried to avoid limitations encountered in the literature. This is especially true of the number of experimental animals. Owing to individual variations, reliable conclusions can be drawn only from the averages of a large number of animals. As a further precaution we have used litter mates of experimental animals for controls.



APPARATUS FOR SMOKING RATS

Fig. 1.

We have used albino rats from the Wistar Institute colony. They have been with us long enough to be perfectly acclimatized. Records of the present colony show that the animals are well fed and are healthy. In all instances young, sexually mature animals were used for experiments. Treatments began from the time of mating and lasted until the weaning of the young.

Careful records were kept of experimental animals and their young. All animals were weighed every second day. Special attention was paid to the length and the course of gestation, to the general health and longevity of the animals, and to the reaction of the mothers toward their young during the time of nursing. The effect of tobacco on the vital functions, and particularly digestion and respiration, was noted. The effect on hair growth was also observed.

The first method was to subject rats to tobacco smoke that would approximate human smoking of about a package of cigarettes a day. For this purpose

an apparatus was devised as shown in Fig. 1. Two holes were drilled in the removable cover of the airtight jar of 6 liters' capacity. One of the holes connected the jar with a smoking cigarette and a bearing-valve rubber bulb was attached to the other. By the operation of the bulb, the chamber of the jar, in which the rats were placed, was filled with as much tobacco smoke as one cigarette could furnish at a time. Only one-third of a cigarette was necessary to fill the jar with smoke. At first three daily exposures were made, but later it was found that the same results were obtained by a daily, three-minute exposure. For the first few seconds of treatment, respiration was noticeably reduced, which, however, returned to about normal so that the animals would make a good many inhalations of the smoke. After a few weeks' treatment the hair became very yellow, due in all probability to the combustion products of the tobacco smoke.

The second method consisted in injection into the rat of solutions of chemically pure nicotine. This method was used to check the efficiency of the first method. Solutions of various strengths were tested, but the final product used was from 1:1,000 to 1:2,000. The injections were made intraperitoneally or subcutaneously, and the amount of the solution used was from 0.5 to 1 c.c. a day per adult rat. Three daily injections were tried at the beginning of the experiment but, as in the case of smoking, this was abandoned in favor of one treatment a day.

The immediate effects on the animals of the two methods are very much the same, except that the injection of nicotine is more severe. Within a minute after injection the animals go into the characteristic nicotine convulsions. The animal stiffens and falls on one side; slight wheezing noises and squeaks are heard, which indicate a type of dyspnea. In a few minutes the violent convulsive condition passes off but the animal still remains on its side or back. If assisted to stand upright, it will fall and roll about, or it may suddenly get up and run aimlessly and fall off the table. The making of rather low sounds or the touching of the animal during the period of recovery will make it jump or go into convulsions again, all of which indicates the reduced threshold of irritability. In some of the treated animals a temporary stage of diarrhea was noted.

In the use of the two methods one may suspect that the mechanics of treatment may be responsible for some of the results. For this reason, some of the control animals were placed in the smoking jar for the usual time and kept there while the air was circulated by the suction bulb. A few of the control animals received injections of neutral distilled water. The results of such treatment were negative in both instances.

RESULTS

Effect on Weight of Young From Treated Mothers.—In this study the young of both smoked and injected mothers were included. Of the controls there were 113 young, from smoked mothers there were 393 young, and from injected mothers 199 young. The large number in each group was purposely selected to avoid objections, often raised in criticism of former experiments, and to balance out individual variations. The latter, as will be shown later, is of considerable importance in this work.

The results are represented graphically in Fig. 2. The horizontal figures represent the weights in grams; the vertical figures, the ages of the rats in days. The condition of the control animals is shown by the broken line. The circles represent the young from the smoked mothers, and the dots, the offsprings of injected mothers. Each circle or dot represents the average weight of one litter. The same is true of the controls plus the fact that the variations have been removed for clarity.

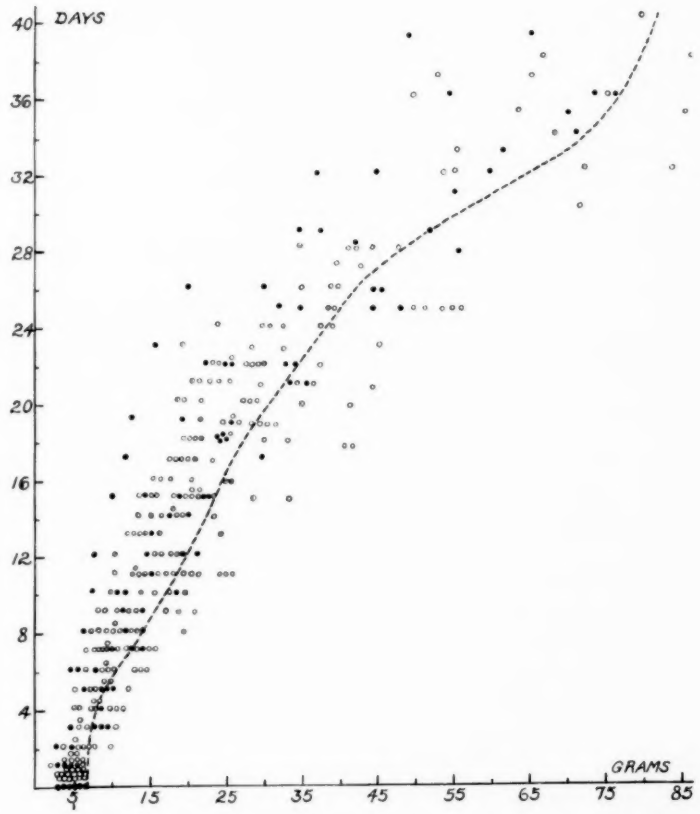


Fig. 2.—Weight record of the young from treated and control mothers. Black dots represent young from injected mothers; circles show young from smoked mothers; broken line denotes young from control mothers. The variations of the litters are omitted for clarity.

It will be seen from Fig. 2 that the young from treated mothers tend to be on the underweight side of the graph. This is true at any given age of the young. Approximately two-thirds of all the young from treated mothers are underweight. The young from injected mothers are slightly more underweight than those from smoked mothers.

Nicotine Injection of Females During Pregnancy and Lactation.—A separate series of animals in litter-mate groups were used for this experiment. Each litter was divided as equally as it was feasible into control and experimental animals, of which there were 33 females in the experimental group and 20 in the control group. In each case treatment of the female began at the time of mating and lasted until the end of lactation. The males were not treated. The nicotine

solution used was 1:1,000. Perhaps the best way to report on these experiments is to present the laboratory records of a representative number of litter-mate groups. The records of five such groups, taken at random, are as follows:

First group of 3 litter mates:

1. Female A1, died after forty-three days of injection; progressive increase of weight up to a certain point indicated pregnancy, but there was no delivery.

2. Female A2, delivered 7 young; raised only 3 beyond seven days; all were underweight.

3. Female A3, increase of weight indicated pregnancy, but there was no delivery; this was followed by extreme emaciation.

Second group of 4 litter mates:

1. Female A23, delivered 5 young; 2 died within five days; all were dead within eight days.

2. Females A24 and A25, showed pregnancy weight increase, but there was no delivery.

3. Female A26, delivered 4 offsprings forty-two days after mating; 2 of these were dead by the third day and the last 2 died on the sixth day.

Third group of 4 litter mates:

1. Female A15, delivered an amorphous fetus and some hemorrhagic material; the female died five days after delivery.

2. Female A16, showed the regular increase in pregnancy weight, but died shortly before the time of delivery; on autopsy the uterus was found to be filled with gangrenous fetuses.

3. Female A17, delivered 5 young; 2 of these were stillbirths and the other 3 died on the fourth day.

4. Female A18, delivered on the forty-sixth day; the young were apparently normal.

Fourth group of 4 litter mates:

1. Female A4 died after twenty-six days of injection; there was no appreciable weight increase; early resorption was suspected.

2. Female A5, delivered 7 young; 5 were dead by the fifth day and all by the ninth day.

3. Female A6, delivered 3 young; 2 survived but were underweight.

4. Female A7, delivered 6 young; 4 survived, all were underweight.

Fifth group of 3 litter mates:

1. Female A8, died during the latter part of pregnancy.

2. Female A9, delivered 5 young; all died within six days.

3. Female A10, failed to deliver young.

Of the 30 control females one failed to deliver. All other litter-mate controls delivered normally and raised healthy young.

The results from all injected females may be summarized as follows:

1. The per cent of females losing one or more of their young before weaning was 63.3.

2. The per cent of females losing all of their young before weaning was 33.3.

Females Exposed to Tobacco Smoke During Pregnancy and Lactation.—Owing to the greater variability of results in this series, a larger number of animals seemed necessary. In all, 74 females were exposed to tobacco smoke. Litter mates of these served as controls. As in the former series, treatment was started immediately after mating. For comparison, the laboratory records of three groups of litter mates, taken at random, are presented.

First group of 4 smoked females:

1. Female B20, delivered 9, raised only 6; all were underweight.

2. Female B21, delivered 6, raised 4; all were underweight.

3. Female B22, gave birth to 8 young, of which 4 died and the remaining 4 were underweight.

4. Female B23, delivered 5 and raised 2, apparently normal young.

Second group of 3 smoked females:

1. Female B4, gave birth to 9 young; all were underweight.

2. Female B5, delivered 5; all were underweight.

3. Female B6, delivered 4; all were underweight and dead by the sixth day.

Third group of 3 smoked mothers:

1. Female B65, delivered 7; one died the first day; all were underweight.

2. Female B66, delivered 4; one died at 21 days of age.

3. Female B67, delivered 7; all were underweight.

From the foregoing records it is apparent that the smoked mothers and their progeny fare considerably better than the injected animals. The results may be summarized as follows:

1. The per cent of females losing one or more of their young before weaning was 28.3.

2. The per cent of females losing all of their young before the weaning period was 13.5.

Females Exposed to Tobacco Smoke Prior to Mating.—This experiment was designed to check the latent effects of tobacco smoke on the mothers. It was desired to treat the females prior to the development of the mammary gland for lactation. If, in so doing, anomalous development of the young can be demonstrated, the injury must reside in the organs other than the mammary gland.

For this purpose 6 females were selected and exposed to daily treatments for three weeks. Upon termination of the treatment, the females were mated and allowed to pass through pregnancy and lactation without further disturbance.

Two litters of young were obtained from each female. The results are summarized as follows:

1. The per cent of females that lost one or more of their young before the time of weaning was 33.3.

2. The per cent of underweight of the young was 25.

Alterations in the Behavior of Treated Mothers.—In untreated rat females a certain amount of inborn maternal "responsibility" toward their young can be observed. Toward parturition a nest, although very crude, is usually made by the mother. The young are kept together in the nest and nourished until they can shift for themselves. If some of the young are removed from the group by the attendant, the mothers are often seen to pick them up and carry them back to the nest. In treated mothers these stereotyped patterns of behavior suffer marked alterations. A nest of any kind is seldom built; the young may be scattered all over the floor of the cage, cold and hungry, without the mother paying any attention to them. There is a noticeable indifference on the part of the mothers toward the nursing of their young. The alterations are more common in the injected females than in the smoked ones. They are more severe within several hours after treatment.

Cannibalism is practically unknown in properly fed rat colonies. None has occurred in the control groups during these experiments. In treated animals, however, cannibalism was rather common. It is the second largest factor in the reduction of the litter size, death due to weakness and maternal neglect being the major factor.

Another peculiarity that must be ascribed to the altered maternal behavior is the neglect on the part of the mothers to sever the umbilical cord after birth of the young. In such instances the umbilicus is left to dry in the young until it breaks off of its own accord or is severed by the attendant. In case the attendant performs the neglected surgical operation, the placenta, which is usually devoured by the mother, is left untouched. The number of such instances is relatively small, but none have occurred in the controls.

DISCUSSION

The weighing of the females and their young every consecutive day appeared, at first, to be very laborious and time-consuming, yet, as the work progressed, it proved to be one of the best diagnostic means at our disposal. Not only the weight differences between the experimental and control animals was established, but also the course of pregnancy and the developmental process of the young could be followed mathematically.

The majority of the underweight young were underweight at the time of birth. Some, however, were of average weight at the time of birth but lost weight during the period of lactation. Similar observations have been made by Nice,[1] Nakasawa,[2] and Hofstätter.[3] Behrend and Thienes[4] injected nicotine into young rats and observed a decrease in weight but no marked retardation in growth. The decrease in weight was found to be due to the lack of fat deposit. Their findings agree with the weight and growth curves of our treated mothers. In the few instances of extreme emaciation of treated females there is reason to believe that it affected more than the fat deposit. The findings of Behrend and Thienes apply to a certain number of the young that were underweight. The lack of adipose tissue deposit was indicated by their skinniness, angularity of body, and wrinkled skin. The majority of the young of

this group did not only show these characteristics but remained smaller in size than the controls. We are forced to conclude that nicotine poisoning varies with the age of the animal.

In these experiments vaginal smears were not taken, and we have no direct evidence as to the conditions of estrus. However, delayed deliveries have occurred in a number of instances. By this we do not mean a delay of four to six days but delays of two or more weeks in duration. The longest delay of delivery on our records is two months. This seems to indicate that an interference in the reproductive cycle has taken place. Interference with the estrus cycle in rats and mice treated to nicotine has been reported by Lee,[5] Unbehaun,[6] and Nakasawa.[2] Since Thienes,[7, 8] Behrend and Thienes,[4] and Staemmler[9] report no effect on the estrus cycle in nicotine treated rats, we assume that estrus disturbances vary with the amount of nicotine administered and the age of the animal in treatment.

Care must be exercised in the appraisal of what we have designated as altered maternal behavior. Apparently this syndrome involves both the mothers and their young. We have found no objective means by which these two can be clearly separated. The disturbances in the digestive, circulatory, and reproductive systems are interpreted as contributory but not major causes of altered behavior of the mothers. Similarly, the interference of nicotine with the development of the mammary glands and lactation, Hatcher and Crosby,[10] Nakasawa,[2] Sodano,[11] and Greiner[12] must be so considered. Since nicotine is primarily a poison of the nervous system (Dixon[13]), and since behavior patterns are chiefly controlled by the nervous system, the major injury must be neural in nature. That this assumption is correct, is indicated by the excitability and restlessness of the mothers and the ease by which they can be thrown into convulsions. That excitability and restlessness afflict most women who have taken to heavy cigarette smoking, is well known to students of the tobacco problem (Campbell,[14] van Hoosen,[15] Greiner,[12] Knopf,[16] and Hofstätter[17]).

Individual resistance to the effects of tobacco smoke offers another point of comparison between treated rat females and smoking human mothers. Some of the litter-mate mothers, receiving the same treatment as the rest, stood the exposure to tobacco products with no apparent detriment. Their young were reared normally, and their weight was normal and sometimes as good as the best of the controls. This was particularly true of the groups that were exposed to tobacco smoke. Likewise there are women who smoke continually and rather heavily and yet they and their offspring remain in what appears to be perfect health. Further investigation will disclose whether these mothers and their offspring are really well or only apparently so.

Nicotine poisoning has been found to occur very often in women employed in the tobacco industry (Klein,[18] Sodano[11]). The case histories of these women are of interest in this connection. Such women are known to suffer from nervousness and insomnia; there are changes in the menstrual cycle and lessened sex desire; they have fewer pregnancies and more abortions and stillbirths; emaciation of the young occurs often, and there is a greater infant death rate.

It will be seen that the parallelism between the histories of women employees of the tobacco industry, and those of our treated rat is strikingly

similar. We interpret that a practical answer to the original question can be found in the case histories of women employed in the tobacco industry and in the histories of women addicted to heavy cigarette smoking.

SUMMARY

The following results were obtained in a study of a large number of nicotine-injected and smoked female rats and their offsprings:

1. Two-thirds of all the young of treated mothers were underweight; the young from nicotine-injected mothers were more underweight than those from smoked mothers.

2. The underweight group remained underweight during the entire period of observation; many of the young of this group were undersized and died early.

3. Of the females injected, 63.3 per cent lost one or more young before weaning, and 33.3 per cent lost all of their young.

4. Of the mothers exposed to tobacco smoke, 28 per cent lost one or more of the young before weaning, and 13.5 per cent lost all of their young.

5. Of the mothers smoked prior to mating, 23.3 per cent lost one or more of their young before weaning, and 25 per cent were underweight.

6. In both groups of the treated mothers, temporary sterility, resorption of young in utero, and abortions were noted.

7. Alteration of maternal behavior was observed and consisted of cannibalism and neglect of the young as to care and feeding.

8. A marked parallelism exists between the treated rat females and their young and human mothers and their young in cases where the mother is a heavy smoker or is employed in the tobacco industry.

9. Individual variation is much in evidence.

REFERENCES

1. Nice, L. B.: Comparative Studies on the Effects of Alcohol, Nicotine, Tobacco Smoke and Caffeine on White Mice, J. Exper. Zoöl. **12:** 133, 1912.
2. Nakasawa, Rukuro: Der Einfluss der chronischen Nicotinvergiftung auf die Funktion der Geschlechtsorgane der weiblichen Ratten, Jap. J. M. Sc. Tr., IV. Pharmacol. **7:** 1, 1933.
3. Hofstätter, R.: Experimentelle Studien über die Einwirkung des Nicotins auf die Keimdrüsen und auf die Fortpflanzung, Virchow's Arch. **244:** 183, 1923.
4. Behrend, Allen, and Thienes, C. H.: Chronic Nicotinism in Young Rats and Rabbits, Effect on Growth and Estrus, J. Pharmacol. & Exper. Therap. **46:** 113, 1932.
5. Lee, Y. C.: Experimental Studies on the Relation Between Nicotine and Sexual Hormone. Part II. The Effect of Nicotine on Morphological and Histological Changes of Female Sex Organs After Injection of Female Sex Hormone, J. Severance Union Med. Coll. **2:** 108, 1935.
6. Unbehaun, Gerd: Untersuchungen über die Einwirkung des Nicotins auf das Ovarium des weissen Maus, Arch. f. Gynäk. **147:** 371, 1931.
7. Thienes, C. H.: Experiments With Nicotine Upon Growing White Mice, Am. J. Hyg. **9:** 500, 1929.
8. Thienes, C. H.: Failure of Nicotine to Alter Estrus Cycle in the White Rat, Proc. Soc. Exper. Biol. & Med. **28:** 740, 1931.
9. Staemmler, M.: Nikotin und Keimdrüsen, München. med. wehnschr. **83:** 658, 1936.
10. Hatcher, Robert A., and Crosby, Hilda: The Elimination of Nicotin in the Milk, J. Pharmacol. & Exper. Therap. **32:** 1, 1927.
11. Sodano, A.: Riserche sperimentali sull' influenza della nicotina sulla funzione genitale della donna, Arch. di obstet. e ginec. **41:** 559, 1934.
12. Greiner, Irene: Nikotinfergiftung, beobachted bei einem Säugling, Jahrb. f. Kinderh. **146:** 131, 1936.
13. Dixon, W. E.: The Tobacco Habit, Lancet **2:** 881, 1927.

14. Campbell, A. M.: Excessive Cigarette Smoking in Women and Its Effect Upon Their Reproductive Efficiency, J. Michigan M. Soc. **34**: 146, 1935.
15. Van Hoosen, Bertha: Should Women Smoke? Motherhood and Tobacco Antagonistic, M. Woman's J. **34**: 226, 1927.
16. Knopf, S. Adolphus: Effect of Cigarette Smoking on Tuberculosis Among Young Women, on the Child in Utero and Early Life, and on Certain Conditions and Diseases in Adults, M. J. & Rec. **130**: 485, 1928.
17. Hofstätter, R.: Über die Schäden des Nikotinmissbrauches, Wien. klin. Wchnschr. **47**: 119, 1934.
18. Klein, S. M.: Über den Einfluss der Tabakindustrie auf Genitalien und Mutterschaft, Arch. f. Frauenk. **17**: 30, 1931.

INSULIN SENSITIVITY OF CATS WITH HYPOTHALAMIC LESIONS AND CATS WITH CERVICAL CORD SECTION*

JOHN R. BROBECK, PH.D., CHICAGO, ILL.

THE role of the hypothalamus in the control of carbohydrate metabolism has been investigated by means of insulin sensitivity tests, in cats by Ingram and Barris,[1] and in cats and monkeys by Cleveland and Davis.[2] Following the placing of hypothalamic lesions with the aid of the Horsley-Clarke stereotaxic instrument, these investigators found the animals more sensitive than normal to the hypoglycemic effect of insulin, and less sensitive than normal to the hyperglycemic effect of adrenalin or the diabetogenic effects of anterior lobe extract. Ingram and Winter[3] have recently mentioned similar insulin hypersensitivity in cats with experimental diabetes insipidus.

During the study of temperature regulation and experimental diabetes insipidus in this laboratory, lesions have been placed in the hypothalami of a number of cats. Insulin tests were performed on 76 of these animals, on 21 normal cats, and on 5 cats in which the spinal cord has been sectioned in the lower cervical region. The procedure for each test was as follows:

For the duration of the test the cat was placed on a canvas hammock and secured there in a side-reclining position by means of an arrangement of web straps. This restraint appeared to cause little discomfort, and animals which had been accustomed to such treatment on several days previous to the insulin test showed little excitement and frequently slept much of the time during the experiment. Control tests showed that during such confinement the blood sugar was relatively constant at a level 10 to 20 mg. per cent higher than the average fasting blood sugar of unexcited cats.

After the cat had been on the hammock for at least ten minutes, a blood sample was drawn from the saphenous vein. A sterile solution of insulin was then injected, usually into the saphenous vein of the other leg, although in certain tests the insulin was given subcutaneously over the saphenous vein. Blood samples were drawn thirty, sixty, one hundred and twenty, and one hundred and eighty minutes after insulin injection, and blood sugars (total

*From the Institute of Neurology, Northwestern University Medical School.
Aided by a grant from the Rockefeller Foundation.
Received for publication, July 31, 1939.

reducing substance) were estimated by the Randles and Grigg[4] modification of the Folin-Wu technique, using 0.5 c.c. samples of blood.

Clotting and fermentation were prevented by the addition of a small amount of a sodium fluoride and thymol mixture (10:1). The dosage of insulin was 0.5 unit per kilogram body weight: the required amount of commercial insulin was diluted with sterile saline solution to a volume of 2.5 to 5.0 c.c., depending upon the size of the cat. Some difference observed in the activity of different brands of insulin led to the selection of Lilly insulin for most of the experiments. The cats were tested after fifteen hours of fasting.

NORMAL TESTS

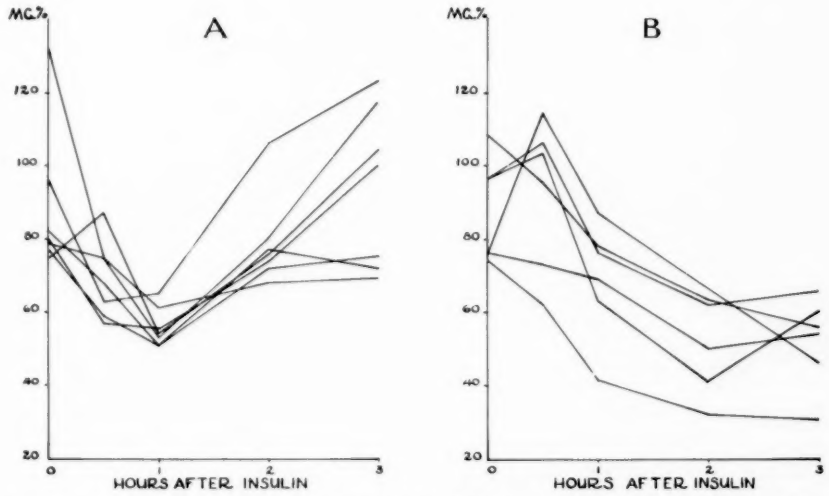
Twenty-two tests were performed on 21 normal cats with 0.5 unit per kilogram of Lilly insulin injected intravenously. In 19 tests the lowest blood sugar level was observed at the end of sixty minutes, while in the other 3 tests the lowest level occurred at the end of thirty minutes. Recovery always took place spontaneously, and in 17 tests the final level was higher than the initial blood sugar. The lowest level noted was 45 mg. per cent; the least amount of final recovery was that of Cat 3 in which the final blood sugar level was 69 mg. per cent, or 87 per cent of the initial level. No external hypoglycemic symptoms were observed in any of the normal animals.

The average initial blood sugar was 94 mg. per cent; the average minimal blood sugar, 57 mg. per cent; the average final level, 109 mg. per cent. At the time of the tests most of these animals had been accustomed to restraint on the hammock.

While this work was in progress a few experiments were carried out with subcutaneous injection of insulin, with results quite different from those obtained when the intravenous method of injection was used. A series of control tests was, therefore, made in which the same animals were tested with insulin injected by both routes, with at least a week's interval between the tests. These experiments showed that hypoglycemia following subcutaneous injection of insulin is more prolonged, that blood sugar reactions are more variable, and that lower levels may be observed than those following intravenous injection of the same amount of the hormone. The results of these tests are presented in Graph 1. From the composite graphs the uniformity between the results of the intravenous experiments is evident, while the blood sugar reactions following subcutaneous injection of insulin were consistent only in that the hypoglycemia was most marked toward the end of the test. Other experiments have suggested that prolonged hypoglycemia may follow the subcutaneous injection of only a part of the dose of insulin when the remainder is then given intravenously.

Three of the 6 cats showed transitory hyperglycemia following subcutaneous administration of the insulin. According to the work of Zucker and Berg,[5] these hyperglycemias may be attributed to an impurity in the insulin; it seems probable that such hyperglycemias also occurred following the intravenous injection of insulin but were undetected in all cases except one, because they occurred in the interval between the first and second samples. Following subcutaneous injection of insulin the hyperglycemic effect was evidently more prolonged.

The cats on which the tests reported in Graph 1 were performed had all been carefully adjusted to handling and confinement on the hammock. The blood sugar averages for the 7 intravenous tests were: initial, 89 mg. per cent; minimal, 55 mg. per cent; final, 94 mg. per cent. The average final level was lower for these 7 tests than for the complete series of 22 tests, suggesting that emotional reactions played a part in the terminal hyperglycemias observed in 17 of the 22 experiments with intravenous insulin. The average amount of hypoglycemia, however, was quite uniform, averaging 55 mg. per cent for the 7 tests of Graph 1, and 57 mg. per cent for the entire series of 22 tests. No external hypoglycemic symptoms were noted following either intravenous or subcutaneous injection.



Graph 1.—Blood sugar responses of normal cats following insulin injection (0.5 u./kg. body weight). *A*. Intravenous injection; *B*. Subcutaneous injection.

Brooks[6] commented on the variability of the insulin sensitivity of normal animals following subcutaneously injected insulin. The more uniform results following intravenous injection make that method of administration preferable when the reaction of an animal to experimentally induced hypoglycemia is to be observed.

CATS WITH HYPOTHALAMIC LESIONS

Insulin tests were performed on 76 cats in which hypothalamic lesions had been produced for the study of temperature control, experimental diabetes insipidus, or carbohydrate metabolism.* Operations were performed under nembutal anesthesia, with the aid of the Horsley-Clarke stereotaxic instrument, using either a unipolar or a bipolar needlelike electrode for the production of electrolytic lesions. Histologic verification of the extent of the lesions was made from 50 micra sections stained by the Weil and cresyl violet techniques, or from 10 micra sections stained with cresyl violet.

*Many of these cats were operated upon by Doctors Magoun, Clark, or Alexander, who kindly allowed their use for insulin experiments.

The results of the insulin tests may be summarized in this way: Thirty-nine animals reacted normally. In 28 animals no hypoglycemic symptoms were observed, but the amount of blood sugar recovery was less than normal; in many cases this could be attributed to postoperative or respiratory infections, or to malnutrition. Hypoglycemic symptoms were observed in 9 cats, associated with inanition, infection, or some other complication in 5 of the 9, but apparently without complication in 4 cats with diabetes insipidus.

TABLE I

INSULIN SENSITIVITY

CATS WITH DIABETES INSIPIDUS

Insulin, 0.5 unit/kg. body weight

CAT NO.	POSTOP. DAY	BLOOD SUGAR (MG. %) MINUTES AFTER INSULIN					NOTES
		0	30	60	120	180	
A 6	83	100	68	58	51	54	Partly subcutaneous
	117	89	69	55	69	99	
A11	100	105	70	65	62		Symptoms of shock
A13	71	122	89	71	37	38	Symptoms of shock
A15	78	86	64	71	70	107	
A16	77	110	85	53	59	44	Symptoms of shock Subcutaneous insulin
A22	106	140	105	77	58	79	
A64	156	127	78	75	69	101	
	162	89	71	60	65	90	
A66	156	88	66	48	48	76	
A70	155	103	83	50	88	115	
A88	62	160	117	73		35*	Symptoms of shock
A90	61	112	38	38	89	120	

*150 minute sample.

Diabetes insipidus: Tests were performed on 11 cats known to have diabetes insipidus as the result of lesions which interrupted the supraoptico-hypophyseal tracts in the anterior hypothalamus (Fisher, Ingram, and Ranson[7]). The insulin experiments were carried out between postoperative days 61 and 162, when the animals were in good physical condition and weighed more than at the time of operation. The data from the tests are reported in Table I. Six of the 11 cats (A6, A15, A64, A66, A70, and A90) were no more sensitive than normal to the hypoglycemic action of insulin, while in Cat A22 recovery was somewhat delayed, although hypoglycemia was not abnormally marked. The remaining 4 cats were hypersensitive; Cats A11, A13, and A88 showed unmistakable symptoms of insulin shock toward the end of the three-hour test period, although the blood sugar level of Cat A11 at the time the symptoms appeared was evidently not as low as the shock level. Cat A16 was tested with subcutaneous injection of insulin and experienced shock shortly after the end of the test; two weeks later, however, a test performed with intravenous injection of insulin gave normal results.

The hypoglycemic symptoms included muscular flaccidity, relaxation of anal and bladder sphincters, and apathy to ordinary visual, auditory, or cutaneous stimulation. Injection of sterile glucose solution either intravenously or subcutaneously promptly relieved the symptoms in all cases, although more than one injection was sometimes necessary to effect complete recovery.

The lesions of Cats A11, A13, A16, and A88 were located in the medial part of the anterior tuberal portion of the hypothalamus in the region close to the ventral extent of the third ventricle where the supraoptico-hypophyseal tracts lie. This corresponds closely with the description of the lesions reported by Ingram and Barris[1] associated with the disturbance of carbohydrate metabolism: "Bilateral and situated either close to the walls of the ventricle in the region of the filiform nucleus or in the perifornical and subfornical regions of the anterior hypothalamic zone."

Other tests: Normal insulin responses were observed after lesions in the anterior portions of the lateral hypothalamic areas, after large transverse lesions at the level of the chiasma, after large midline lesions between chiasma and infundibulum, after discrete bilateral lesions in the fornices, or after discrete lesions in the midline at the level of the median eminence. There was no correlation between disturbance of temperature regulation and insulin responses, and cats in which the paraventricular nuclei were almost completely destroyed reacted as normal.

TABLE II

BLOOD SUGAR FOLLOWING HYPOTHALAMIC LESIONS

WEEK NO.	TOTAL TESTS	NO. OF CATS	BLOOD SUGARS IN MG. %		
			MINIMUM	MAXIMUM	AVERAGE
Preop. control	45	41	65	133	88
1	15	15	83	170	108
2	26	25	65	119	88
3	20	20	69	110	85
4	17	16	67	119	81
5	17	15	67	100	79

From this series of experiments it was not possible to assign to any hypothalamic structure definite function in the control of carbohydrate metabolism. The 4 hypersensitive animals showed similar lesions in the medial portion of the anterior tuberal hypothalamus, but other cats with lesions in the same area reacted normally to insulin; and one animal with destruction of almost the entire anterior hypothalamus reacted normally when tested 19 days after the second stage of a two-stage hypothalamic operation.

To supplement the data obtained from insulin tests, estimations were made of fasting (fifteen hours) blood sugar levels of some of these animals both before operation and for a period of 5 weeks after operation. In Table II the data from these analyses are arranged according to the week in which the tests were performed, with the preoperative control tests listed at the top of the series. From a preoperative average of 88 mg. per cent, the blood sugars of the cats rose to an average of 108 mg. per cent during the first postoperative week and then gradually fell to 79 mg. per cent at the fifth week after operation. The immediate postoperative rise may be attributed to the effect of the operations, for the minimal as well as the maximal blood sugars noted during the first week were higher than the control levels. On the other hand, the ultimate decline of the average levels to a figure 9 mg. per cent lower than the control

average does not represent a tendency toward spontaneous hypoglycemia, for the minimal levels were almost the same before operation as during the second, third, fourth, and fifth postoperative weeks. The lower average was coincident with a similar decline in the maximal levels observed, and probably represented a tendency toward emotional stability in the cats as they became more accustomed to being handled and placed on the hammock. As a result, five weeks after operation there was less emotional hyperglycemia associated with the taking of the samples. These data confirm the observation of postoperative hyperglycemia made by Barris and Ingram[8] following the production of hypothalamic lesions, but during the first five postoperative weeks this series of cats did not show the hypoglycemia which they described.

SPINAL CATS

Insulin tests were performed on spinal cats by Brooks,[6] but the animals he used were operated upon only a week previous to the tests and were not eating readily at the time they were utilized for the experiments. His results, however, indicated that such cats were able to recover normally from small doses of insulin.

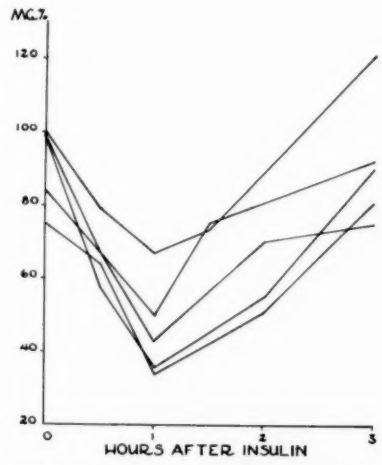
This observation has been confirmed by a series of insulin experiments made on 5 cats in which the spinal cord had been sectioned in the lower cervical region, the level of section varying from the roots of C_6 to the caudal roots of C_8. The animals were operated upon under ether anesthesia, a segment of the cord was actually removed, and the dry operative field was carefully examined to assure removal of a complete section.* Histologic examination of tissue from similarly operated cats has shown complete interruption of all fibers in every case.

Tests were performed three to five weeks after operation when all the cats were eating well and appeared to be in good physical condition. The dosage of insulin was 0.5 unit per kilogram body weight, injected intravenously, and the cats were fasted for fifteen hours before the tests.

Following this amount of intravenous insulin, blood sugar recovery took place normally. The average initial level was 91 mg. per cent; the average minimal level, 46 mg. per cent; and the average final level, 92 mg. per cent. The average initial and final levels compare well with those of the 7 normal intravenous tests of Graph 1, but the average minimal level was somewhat lower for the spinal cats (46 mg. per cent) than for the normal animals (55 mg. per cent). Two of the spinal cats showed hypoglycemic levels well within the normal range (67 mg. per cent, Cat K153; 50 mg. per cent, Cat K157), although the abnormally low levels of hypoglycemia of Cats K158 and K161 brought the general average 9 mg. per cent below that of normal cats. The significance of this difference is not known, although it may be attributed to disconnection of the thoracolumbar outflow of the autonomic system from the higher centers of medulla and hypothalamus. The ultimate blood sugar recovery was as good in the spinal as in the normal animals. Data from these tests are represented in Graph 2.

*The animals were operated upon by Doctors Magoun and Clark, and were cared for by Dr. Clark.

Although external signs of hypoglycemia were not observed in any normal cats, such symptoms were marked in 3 of the 5 spinal animals. Coincident with the time of most marked hypoglycemia, the respiratory rate of Cat K153 rose to 300 per minute, of Cat K157 to 264 per minute, and of cat K158 to 360 per minute. Panting was vigorous and sustained, there was increased salivation, muscular flaccidity, and apathy to stimulation to such a degree that the state of the animals resembled mild coma. These symptoms were shown to a lesser degree by Cat K161, but Cat K155 appeared and reacted as normal throughout the course of the test, although its hypoglycemia was as marked as the others. The respiratory rate of K155 never rose above 28 per minute.



Graph 2.—Blood sugar responses of spinal cats following intravenous insulin injection (0.5 u./kg. body weight).

There was no apparent correlation between the appearance of these external symptoms and the blood sugar reactions of the different cats, with respect either to the severity of the hypoglycemia or the amount of ultimate restoration. Spontaneous recovery from the symptoms took place as the blood sugar returned to normal levels.

DISCUSSION

Brooks[6] found that spinal animals were able to restore their blood sugar level to normal following doses of insulin as large as 0.5 unit per kilogram body weight given subcutaneously, whereas following larger doses many of the animals did not spontaneously recover. His work further indicated that the thoracolumbar outflow was capable of some functioning without connection with higher centers, since spinal animals in which the adrenals had been inactivated by removal of one and denervation of the other died from 0.5 unit per kilogram body weight dosage. Hypoglycemic symptoms which he observed included mewing, salivation, pupillary dilatation, and defecation; and "diaphragmatic" panting was frequently noted.

The data reported here confirm Brooks' work and compare directly the blood sugar changes induced by insulin in normal and spinal cats which were well recovered from the effects of the operation.

The insulin tests on spinal cats, however, furnish no explanation of the hypersensitivity to insulin which follows some hypothalamic lesions. With the same dose of insulin from which the spinal cats recovered, 4 cats with hypothalamic lesions showed marked symptoms of insulin shock, and 2 of the 4 (Cats A13 and A88) clearly showed prolonged and severe hypoglycemia. Ingram and Barris[1] observed hypoglycemic symptoms in cats with hypothalamic lesions following administration of a smaller dose of insulin, 0.5 unit total dose. Cleveland and Davis[2] likewise noted hypersensitivity to insulin using only 0.25 unit per kilogram body weight. Since spinal cats recovered normally from doses of insulin which produced dangerous reactions in certain cats with hypothalamic lesions, it seems unlikely that the hypersensitivity of the latter was due to destruction of any center normally acting by way of the thoracolumbarsacral portions of the cord.

Another hypothesis has been suggested by the fact that the insulin hypersensitivity appeared in animals with experimental diabetes insipidus (see Ingram and Barris,[1] and Ingram and Winter[3]), which the work of this laboratory has shown to be due to atrophy of the pars nervosa of the hypophysis (Fisher, Ingram, and Ranson[7]). The antagonism between the action of insulin and posterior lobe extracts has been established by Burn,[9] Geiling, Britton and Calvery,[10] Houssay and Potick,[11] Magenta,[12] Houssay and Magenta,[13] and Ellsworth.[14] It seems reasonable to suppose that atrophy of the posterior lobe following hypothalamic lesions might leave the activity of insulin relatively unopposed. Our series of experiments, however, included cats which showed marked polyuria and polydipsia, yet which reacted normally to insulin, indicating that the diabetic symptoms and insulin hypersensitivity are not necessarily related.

A third possible explanation has been adopted by Ingram and Winter[3] who believe that the insulin hypersensitivity is due to suppression of the function of the anterior lobe; this supposition cannot at present be either proved or disproved. Insulin tests have been performed on monkeys in which the hypophyseal stalk was sectioned just proximal to the dorsal surface of the gland; but the results of the tests were normal (Brobeck, Magoun, and Ranson[15]). Interruption of fibers from the hypothalamus entering the infundibular process and pars distalis by way of the infundibular stalk, therefore, did not result in insulin hypersensitivity.

It seems probable that in some instances the abnormal sensitivity to insulin which appears to follow the production of hypothalamic lesions may be due simply to malnutrition, postoperative or respiratory infections, or inadvertent subcutaneous injection of a portion of an intravenous dose of insulin. No clear explanation has been found, however, for the hypersensitivity which was observed in 4 cats with diabetes insipidus and which has been reported by other investigators.

SUMMARY

Following subcutaneous injection of insulin in normal cats, hypoglycemia was more prolonged and in some instances more marked than that following intravenous injection of the same amount of insulin.

Normal fasting blood sugars and normal insulin reactions were noted in a large number of cats with lesions in various portions of the hypothalamus.

Insulin hypersensitivity was observed in 4 cats with lesions in the medial part of the anterior tuberal portion of the hypothalamus.

Spinal cats recovered as well as normal cats from 0.5 unit of insulin per kilogram body weight, injected intravenously.

REFERENCES

1. Ingram, W. R., and Barris, R. W.: Evidence of Altered Carbohydrate Metabolism in Cats With Hypothalamic Lesions, Am. J. Physiol. **114:** 562, 1936.
2. Cleveland, D., and Davis, L.: Further Studies on the Effect of Hypothalamic Lesions Upon Carbohydrate Metabolism, Brain **59:** 459, 1936.
3. Ingram, W. R., and Winter, C. A.: Blood Sugar in Cats With Diabetes Insipidus Before and After Adrenalectomy, Proc. Soc. Exper. Biol. & Med. **41:** 449, 1939.
4. Randles, F. S., and Grigg, W. K.: Estimation of Blood Sugar by Folin-Wu Method Using 0.1 c.c. of Blood, J. A. M. A. **82:** 684, 1924.
5. Zucker, T. F., and Berg, B. N.: The Course of Blood Sugar After Intravenous Insulin in Normal Dogs and Cats, Am. J. Physiol. **119:** 531, 1937.
6. Brooks, C. M.: The Resistance of Surviving Spinal Animals to Hypoglycemia Induced by Insulin, Am. J. Physiol. **107:** 577, 1934.
7. Fisher, C., Ingram, W. R., and Ranson, S. W.: Diabetes Insipidus and the Neuro-Hormonal Control of Water Balance: A Contribution to the Structure and Function of the Hypothalamico-Hypophyseal System, Ann Arbor, 1938, Edwards Brothers, Inc.
8. Barris, R. W., and Ingram, W. R.: The Effect of Experimental Hypothalamic Lesions Upon Blood Sugar, Am. J. Physiol. **114:** 555, 1936.
9. Burn, J. H.: The Modification of the Action of Insulin by Pituitary Extract and Other Substances, J. Physiol. **57:** 318, 1923.
10. Geiling, E. M. K., Britton, S. W., and Calvery, H. O.: The Modification of Insulin Action in Medulliadrenal Inactivated Cats by Post-Pituitary Extracts, J. Pharmacol. & Exper. Therap. **36:** 235, 1929.
11. Houssay, B. A., and Potick, D.: Antagonisme entre l'hypophyse et l'insuline chez le crapaud, Compt. rend. Soc. de biol. **101:** 940, 1929.
12. Magenta, M. A.: Action des diverses substances hypophysaires sur l'effet de l'insuline, Compt. rend. Soc. de biol. **102:** 428, 1929.
13. Houssay, B. A., and Magenta, M. A.: Action des substances rétro-pituitaires sur la sensibilité à l'insuline des chiens privés d'hypophyse, Compt. rend. Soc. de biol. **102:** 429, 1929.
14. Ellsworth, H. C.: The Antagonism Between Posterior Lobe Pituitary Hormones and Insulin, J. Pharmacol. & Exper. Therap. **56:** 417, 1936.
15. Brobeck, J. R., Magoun, H. W., and Ranson, S. W.: Insulin Sensitivity of Monkeys After Section of the Hypophyseal Stalk, Proc. Soc. Exper. Biol. & Med. **42:** 622, 1939.

ANIMAL TISSUE REACTION TO PARTICULATE COPPER STEARATE*

PHILLIP T. KNIES, COLUMBUS, OHIO

THE response of animal tissue to the introduction of particulate matter has been described principally with reference to silica or silicates in finely divided form. So characteristic a picture has resulted from contact with these materials both in experimental animals and in the human organism in certain occupations that the resulting syndrome has been distinguished by name from other pneumonoconioses which present different or less characteristic clinical, roentgenologic, and pathologic features.

Those characteristics of silicosis which have warranted distinction from reactions to most other particulate matters are the cumulative nature of the tissue changes resulting from continued exposure, and their progression, even after contact with the causative agent has been interrupted. More or less distinctive tissue reactions have been considered relative to numerous other substances including barium,[1] carbon,[8] sulfur,[5] iron,[8] beryllium,[3] titanium oxide,[16] zinc,[21] and aluminum oxide.[15] Some organic dusts have been considered to lead to less typical and usually more acute reactions, such as shoddy fever and thresher's fever. With most such substances, however, the progressive and cumulative nature of the tissue reactions has been less notable than acute exudation or mere mechanical accumulation of the dust. In most instances in which important chronic tissue changes result from exposure to nonsiliceous material, they may be shown to be dependent upon contaminating silica or silicates, which independently exert their characteristic effects.

The possibility that copper in the form of the stearate when introduced in particulate form might give rise to a characteristic clinical and pathologic picture was suggested by the finding of marked pulmonary fibrosis in a patient whose history indicated heavy industrial exposure to copper stearate dust, and in whom no likelihood of inhalation of silica was apparent. Furthermore, the clinical and roentgenographic features of this patient were different in some respects from those usually encountered in silicosis.

CASE HISTORY

A. P., a 46-year-old white male, was admitted to the Medical Service of Starling-Loving Hospital, complaining of dyspnea, dependent edema, and enlargement of the knees. His occupation had included farming and lumbering, and four years before admission for six months, the hauling of washed, wet river gravel and sand, the dust of

*From the Department of Medicine, Ohio State University School of Medicine.
Received for publication, July 21, 1939.

which he insisted was negligible. He was well until two years before admission, when he was employed for nine days cleaning scale from a large copper vat, in which stearic acid had been prepared. The scale had dried and cleaning was accomplished by a motor-driven rotary wire brush. He used no mask and inhaled much dust, with the prompt development of a sore throat and cough. Edema of his feet and ankles soon appeared, with dyspnea on exertion which prevented work, though he remained ambulatory. These complaints became progressively severe with the production of a slight sputum that was repeatedly negative for tubercle bacilli. Within the past year curvature of the nails and clubbing of the fingers had appeared, with a slight soreness of the long extremital bones, and the loss of 20 pounds. Fatigability and precordial palpitation had become marked.

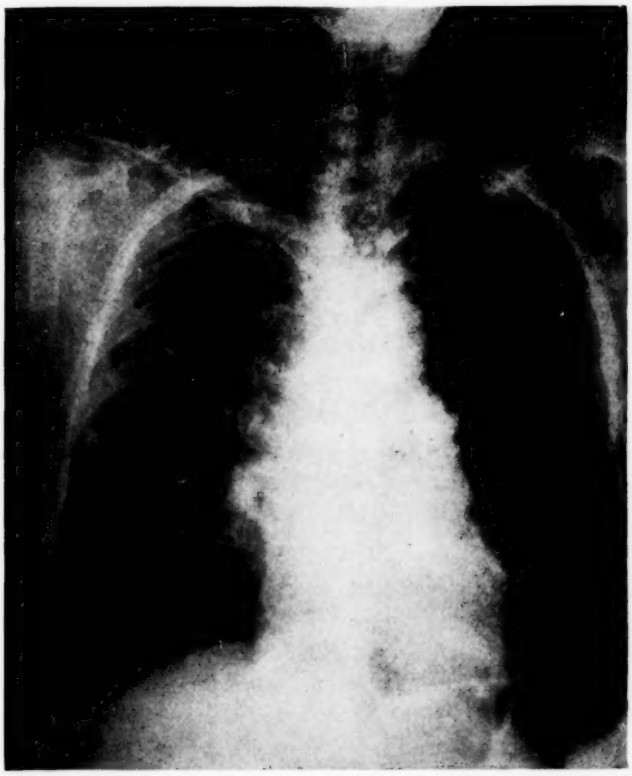


Fig. 1.—Roentgenogram of chest of patient presenting symptoms of pulmonary fibrosis and emphysema following intense exposure to copper stearate dust. Note reticulated fibrosis throughout all lobes of both lungs with emphysema.

On admission there was moderate cyanosis of the lips, with hyperpnea and dyspnea on slight activity. There was rounded prominence of the anterior tibial margins, and enlargement of the wrists, hands, ankles, and feet. The right axillary and both inguinal groups of lymph nodes were slightly enlarged, though discrete and soft. The heart was enlarged to percussion; sounds were distant, and there were no murmurs. The pulmonic second sound was moderately accentuated. Rhonchi were heard throughout both lungs, and coarse râles in both bases. Blood pressure was 110/72. The liver was palpable, but the abdomen was otherwise normal. Enlargement of and grating in both knees on motion suggested localized hypertrophic arthritis.

Biopsy upon a right axillary node revealed almost absent germinal centers, and slight hyperplasia of the reticulum; however, Hodgkin's disease could not be identified. The

chest roentgenogram (Fig. 1) showed a marked diffuse pulmonary fibrosis, suggestive of pneumonoconiosis, but consisting in a fibrous reticulation rather than the nodular infiltration of typical silicosis. There was no area of pneumonic consolidation or of excavation. X-rays of the long bones showed the periosteal thickening of hypertrophic pulmonary osteoarthropathy. Serologic reactions for syphilis were negative, and the blood cytology and urinalysis were normal. The patient was discharged unimproved after a nine-day afebrile course.

LITERATURE AND MATERIALS

The toxicity of copper in the animal organism has been recognized, and has been held responsible for hemolysis and for development of a pigmentary hepatic cirrhosis. The possibility that absorption of the metal sufficient to induce such toxic results might follow the inhalation of particulate copper or copper-containing dust has also been pointed out.[19]

The development of a characteristic pulmonary clinical picture following the inhalation of finely divided copper or copper compounds was reported in 1887 by Lattimer[13] under the term "copperman's chest," a name applied by the laity to a disability among workers in copper smelters, particularly in the pulverizing and molding departments. The disease apparently consisted of bronchitis, emphysema, bronchiectasis, pulmonary congestion, and heart failure, with signs and symptoms of these entities. A brass-founders' ague was noted in some, but it may have been due to plumbism. A progressive pulmonary fibrosis was noted in all cases of the malady reported. The recognized contamination of the ores by iron, sulfur, tin, arsenic, and "earthy matters," however, rendered uncertain any conclusions regarding the pathogenicity of the copper itself and suggested the possibility of concomitant silicosis.

Murray in 1900 reported similar pulmonary changes with laryngitis, chest pain, dyspnea, and cough among workers in brass, which he preferred to attribute to copper rather than to lead.[19] Pneumonoconiosis and a short working life were noted by Landis among the Butte, Montana, copper miners and smelters in 1916,[12] but were ascribed to silicosis. Hayhurst[10] and Hamilton[9] considered that of the "various afflictions" of copper miners "none can be charged to any peculiar toxicity of copper itself," and that "the ill health found among copper miners and workers . . . is caused by something else" such as lead, arsenic, free silica, carbon monoxide, or heat-fatigue toxins.

More recently pulmonary changes in copper workers have been reported by Waetgen[23] and by Arustamova,[2] but again not under circumstances eliminating the confusing possibilities of tuberculosis and silicosis. It is notable, however, that of the sixteen presumably tuberculous copper miners observed by Arustamova, no single instance was included of positive sputum or of pulmonary excavation. Such factors are compatible with a predominant role of silicosis in the production of the clinical picture. This is also indicated by the recovery of silica from the ashed lungs of these miners to an extent of 40.6 per cent of the insoluble residue; calcium and iron compounds were also found in notable percentages, but only a trace of copper could be demonstrated. Such reports are suggestive that the progressive and cumulative

effects of the inhalation of copper-containing dusts in the persons reported were due principally to the contaminating silica or silicates, but critical interpretations were not possible and animal experimentation was not made.

Accordingly, it was intended in the present experiments to determine the pathogenicity of copper compounds when introduced into animals in particulate form, especially in terms of their capacity to elicit tissue fibrosis and chronic foreign body reaction independently of contaminants. In view of the cited case history, copper stearate was employed for this purpose. The toxicity of this compound, used industrially in antifouling paints for the bottoms of vessels, has been recognized,[6] and its employment as a fungicide has been suggested.[14]

The copper stearate used was a fine green blue powder, light in weight, and graded by air flotation to particles with maximum diameters of 2 to 10 microns. These were fragments of monoclinic crystals, soluble in turpentine oil, chloroform, and benzol, but insoluble in water. Analysis showed a strong trace of iron, and an insignificant trace of silica, possibly resulting from the glassware used. Iodine number determination showed approximately a 3 per cent contamination with copper oleate. In the suspension of this material in saline for use in the present experiments sodium stearate was used in concentrations of approximately one part to one thousand of solution, to facilitate wetting the powder and to render the suspensions more permanent.

The copper stearate was introduced into guinea pigs by intraperitoneal injections and by dusting in a cloud chamber, as recommended by McCord[17] and by Haynes[11] for the determination of the pathogenicity of a finely divided substance. Such contacts have been shown to give rise to immediate irritative reactions to most highly insoluble particulate material regardless of its identity. Of the ultimate reactions, however, five general types are recognized by McCord:

(1) Absorptive, with disappearance of the dust without reaction or scar tissue.
(2) Inert, the dust remaining unchanged, without reaction.
(3) Proliferative, with nodules of newly-formed fibrous tissue, increasing in size so long as the stimulating material is undissolved.
(4) Mixed reactions with mixed dusts.
(5) Miliary proliferation following mechanical dissemination of the substance by the acute exudative reaction.

A tissue response following exposures to copper stearate might be due to either the copper or the stearic acid radicals. The accumulation in guinea pigs of inhaled metallic copper dust has been shown by Remy[20] to be principally in the liver and spleen, and to a lesser extent in the lungs, without apparent local damage. "It could also be shown that within a few weeks . . . the increased copper was decreased, and the proper (normal) amount again appeared" on analysis of the individual tissues. The elimination from the blood within twenty-four hours of either ionic or colloidal forms of copper has been demonstrated by Yano,[24] with ultimate excretion of the metal in the bile rather than the urine.

The possible pathogenicity of the stearic acid radical was suggested by the identification of 10-methyl stearic acid among the tuberculolipids,[22] and by the specific tissue reactions to lipids noted by Fallon.[4] The unlikelihood of a specific tissue response to stearic acid was forecast, however, by the complete absence of a typical pulmonary reaction from the industrial medical files of a large soap and stearic acid manufacturer over a period of many years.[14] The condition of the present experiment, of course, permits evalution of only the entire compound, copper stearate.

EXPERIMENT

To evaluate the pathogenicity of copper stearate in the present experiments, four series of animals were used. The first group of five guinea pigs received 100 mg. of copper stearate in saline suspension intraperitoneally through a gauge 20 needle introduced with local antisepsis through the rectus muscle. This dose was considered the first of a series of similar injections, but the entire group died within two hours after treatment, thus furnishing a measure of the immediate toxicity of the material and affording an opportunity to study the pathology of the acute poisoning. A second group of eight animals received from one to ten intraperitoneal injections at three- to four-day intervals of 25 or 50 mg. of copper stearate, and were sacrificed at from twenty minutes after the first injection to fifteen weeks after the last of eight injections. Upon death by chloroform anesthesia particular attention was paid to the omentum, the mesentery, and the abdominal wall in which some suspension was unavoidably deposited at each injection. The histopathology of these structures is reported below.

The third series of eight animals received a single heavy dusting of ten minutes' duration in a chamber with a motor-driven fan[10] and were sacrificed at intervals of thirty minutes to six months thereafter, thus permitting observations of the progressive or recessive character of the resulting tissue reactions. A fourth series of four animals was sacrificed at intervals during and after five months of frequent dustings, to permit the evaluation of the cumulative characteristics of the tissue reactions; three of these animals received 110 dustings each.

INTERPRETATION OF HISTOPATHOLOGY

In the interpretation of these tissue reactions it is important to recall the peculiarities of the guinea pig lung in which small areas of apparent congestion and of alveolar epithelial proliferation are fairly common, as also small lymph follicles about blood vessels, adjacent to bronchi, and scattered throughout the parenchyma, often infiltrated with eosinophiles. The recognition of intercurrent and of parasitic pulmonary disease is also important.

All animals injected intraperitoneally showed local reaction and absorption as well as reaction in the lymph nodes. Those which died early showed slight evidence of vasoparalysis and moderate liver damage; all had abscesses of the abdominal wall and fibrosis of the mesentery. The adrenal glands of the dead animals were depleted of lipoids, and the liver was deficient in glycogen. The lungs of some animals showed pneumonia of doubtful relationship to this experiment, and one animal showed pulmonary atelectasis

with extensive pneumonitis, which may have been an incidental process. Negative polymorphonuclear chemotaxis was suggested by the minimal numbers of these cells, even at the sites of abscess formation (Figs. 2 and 3).

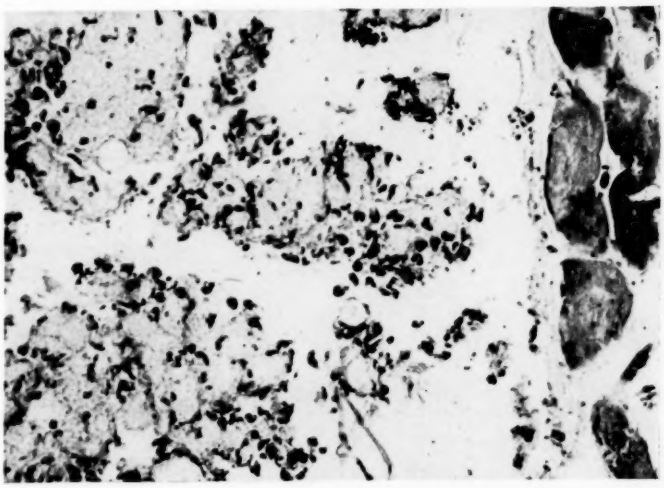


Fig. 2.—Section of abdominal wall of GP, dead after fifth daily injection of 5 mg. of copper stearate in suspension intraperitoneally and subcutaneously, showing presence of crystals with phlegmon and abscess formation. (Hematoxylin eosin ×375.)

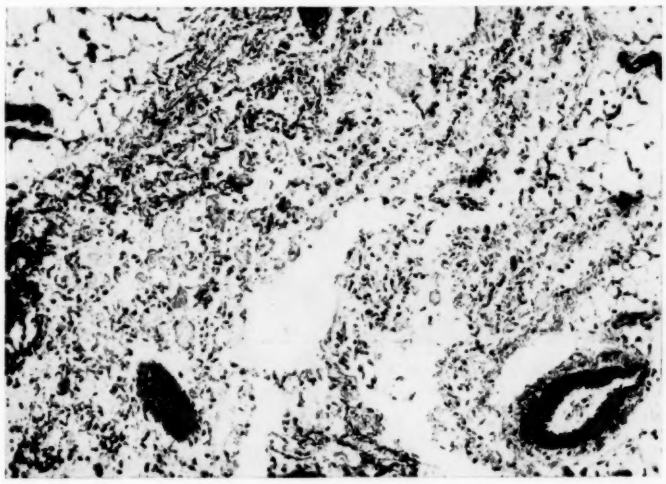


Fig. 3.—Section of omentum showing presence of copper stearate crystals in fat tissues with slight leucocytic infiltration. GP died two hours after intraperitoneal injection of 100 mg. of copper stearate suspension. (Hematoxylin eosin ×180.)

Those animals which survived and were killed by chloroform were remarkably well three months after the injections; the only pathology remaining was a slight fibrosis of the spleen and lymph nodes, with pigmentation of uncertain origin. There was no chronic liver or kidney damage. The local areas of acute reaction had apparently healed with pigmentation and slight fibrosis.

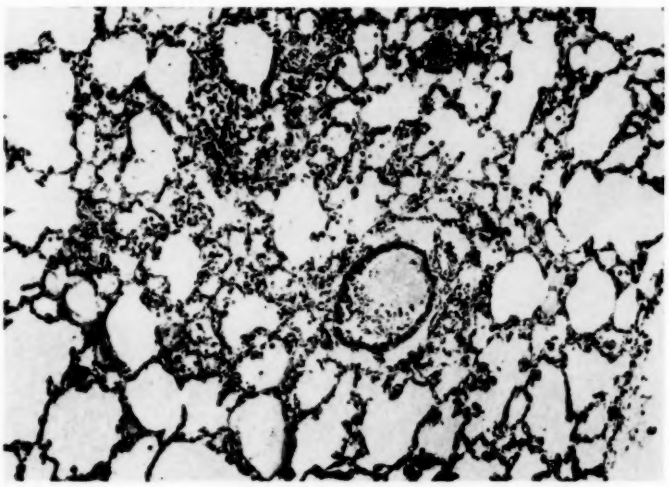


Fig. 4.—Section of lung of GP dead one day after single heavy dusting, showing leucocytic infiltration and edema. Desquamated epithelium and crystals fill the bronchus which is surrounded by atelectasis and compensatory emphysema. (Hematoxylin eosin ×180.)

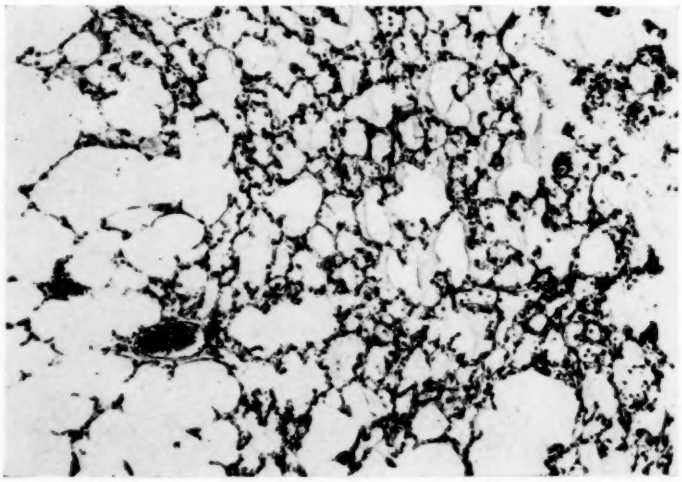


Fig. 5.—Section of lung of GP dead thirty minutes after single heavy dusting, showing acute pulmonary edema with leucocytic immigration and compensatory emphysema. (Hematoxylin eosin ×180.)

The dusted animals revealed an acute injury to the mucosa of the bronchi, leading to desquamation and necrosis of epithelium, and exudation into the lungs. This apparently produced a partial atelectasis, with compensatory emphysema (Fig. 4). One animal died with acute pulmonary edema, suggesting anaphylactic shock (Fig. 5). At this period small areas of interstitial pneumonia could be found. If killed later the acute bronchial reaction had disappeared, but there remained irregularity of air distribution throughout the lungs.

If dusted repeatedly there was apparently not much cumulative effect. One animal died with intercurrent infection; the others showed a few scat-

tered nodules of histiocytes and leucocytes, and some undefined pigment in the pulmonary septa (Fig. 6). The bronchi were normal but there was some irregular subpleural infiltration. None of these changes could, however, be considered a severe impairment of lung function. The other organs were normal in the dusted animals except that the splenic pigment was often slightly increased and one animal showed a chronic infectious splenic tumor.

Thus all experiments both by injection and by dusting showed an acute necrotizing focal reaction, which had subsided within a month, and in no case was there evidence of progression or cumulation in the cellular reaction.

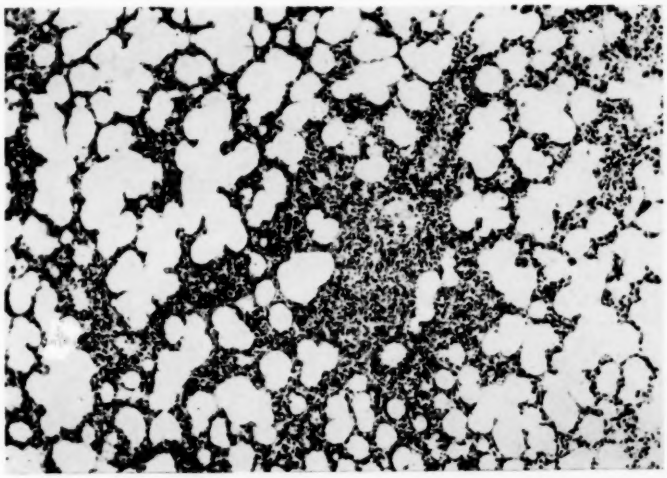


Fig. 6.—Section of lung of GP killed after numerous exposures over three months' time, showing nodular leucocytic and histiocytic infiltration and thickening of septa. (Hematoxylin eosin ×180.)

DISCUSSION

Such observations would suggest the classification of copper stearate in the absorptive group of particulate materials as defined by McCord, with complete disappearance of the injected material after the acute reaction, without notable permanent change or scar tissue. This experiment, therefore, affords no support to an assumption that the reticulated pulmonary fibrosis of the clinical case reported here constituted a specific reaction to inhalation of copper stearate. The fundamental differences in structure of the lungs of guinea pigs and of man are recognized but appear of minor importance to the present experiment in view of the very similar reactions obtained in both to pathogenic particles of silica or silicate. The period of ten to fifteen minutes' heavy dusting to which the third group of animals was subjected, is by direct proportion equivalent to five to eight hours' dusting of the human animals, considering the relationships of their normal life spans; the greater intensity of dust encountered by the animals even further equalizes their exposures to that of the case reported, so that the present experiment would appear adequate to permit comparison of results.

SUMMARY

1. The case is reviewed of a patient presenting marked diffuse pulmonary fibrosis of an unusual reticulated nature, under circumstances suggestive of pneumonoconiotic origin, following the inhalation of copper stearate dust.

2. Several references in the literature recognize the incidence of pulmonary fibrosis among copper workers involving the variables of copper, tuberculosis, silicosis, and other mineral pneumonoconioses.

3. Animal experiments were conducted to determine the tissue responses to copper stearate particles introduced subcutaneously, intraperitoneally, and by inhalation.

4. Such experiments demonstrate an acute reaction to copper stearate and its ultimate absorption and removal without residual or progressive chronic changes.

5. There has thus been afforded no experimental basis upon which to conclude that the pulmonary fibrosis noted in the patient was due to the copper stearate dust inhaled.

40 South Third Street

REFERENCES

1. Arrigoni, A.: Pneumoconiosis Due to Barium, Med. d. lavoro **24**: 461, 1933.
2. Arustamova, A. T.: Pneumonoconiosis Among Workers in Copper Mines, Bor. s. tuberk. **10**: 331, 1932.
3. Marradi, Fabroni S.: Atypical Pneumonoconiosis Due to Beryllium Dust, Med. d. lavoro **26**: 297, 1935.
4. Fallon, J. T.: Specific Reactions to Phospholipids, Canad. M. A. J. **34**: 223, 1937.
5. Ferrannini, A.: Pneumoconiosis in Sulphur Workers, Rassegna di med. appl. lavoro indust. **2**: 359, 1936.
6. Gardner, H. A., and Coleman, R. E.: Metallic Soaps in Paint and Varnish Making, Amer. Paint and Varnish Mfg. Assoc., Circ. 122-124, 1914-1921, p. 30.
7. Gardner, L. U.: Pathological Reactions in Various Pneumoconioses, J. A. M. A. **101**: 594, 1933.
8. Haines, J. B.: Dangerous Dusts, New England J. Med. **216**: 162, 1937.
9. Hamilton, L.: Industrial Poisons in the United States, New York, 1925, Macmillan Co., p. 276.
10. Hayhurst, E. R.: Copper and the Copper Industry, Philadelphia, 1924, P. Blakiston's Son & Co., pp. 321-327.
11. Haynes, S.: Experimental Dust Inhalation in Guinea Pigs, J. Hyg. **31**: 96, 1931.
12. Landis and White: Miner's Circular 20, U. S. Bur. Mines, 1916.
13. Lattimer, H. A.: Chest Diseases Affecting Workers at Copper Work, Lancet **1**: 1126, 1887.
14. Lees, A. H.: Copper Stearate, Annual Rep. Bristol Agric. and Horticult. Research Station, p. 39.
15. Lemon, W. S., and Higgins, G. M.: Pulmonary Lesions Experimentally Produced by Intratracheal Introduction of Aluminum Oxide and of Borosilicate-Glass, Am. Rev. Tuberc. **30**: 548, 1934.
16. Lenzi, L.: Pneumoconiosis Due to Titanium Oxide, Rassegna di med. appl. lavoro indust. **7**: 301, 1936.
17. McCord, C. P., Fleming, R. L., Ainslee, H., and Johnston, J.: Harmfulness of Dust, Surg., Gynec. & Obst. **63**: 129, 1936.
18. Mavrogordato, A.: Construction of Dusting Changers, J. Hyg. **17**: 439, 1918.
19. Murray, W.: Chronic Brass Poisoning, Brit. M. J. **7**: 1334, 1900.
20. Remy, E.: Resistance of Guinea Pigs to Tubercle Bacilli After Treatment With Copper Dust, Ztschr. f. Immunitätsforsch. u. exper. Therap. **78**: 173, 1933.
21. Schmidt, H.: Disease of Lung Caused by Zinc Dust in Galvanizing Industry, Zentralbl. f. Gewerbehyg. **13**: 296, 1936.
22. Spielman, M. A.: Chemistry of Lipids of Tubercle Bacilli, J. Biol. Chem. **106**: 87, 1934.
23. Waetgen, J. W., Volfoth, S., and Jeager, W.: Copper Dust in Miner's Lungs in Mansfield Mines, Arch. f. Gewerbepath. u. Gewerbehyg. **2**: 688, 1931.
24. Yano, Y.: Dispersion and Excretion of Colloidal Copper and Organic Copper Compounds, Folia pharmacol. japon (Brev.) **25**: 29, 1938.
25. Zuemer, E. T.: Personal communication from Procter and Gamble Co., Jan. 6, 1937.

THE DISTRIBUTION OF SULFANILYL-2-AMINOPYRIDINE IN THE BODY*

HERMAN CHINN, PH.D., AND JOHN BELLOWS, PH.D., M.D.
CHICAGO, ILL.

DESPITE the widespread clinical use of sulfanilyl-2-aminopyridine, very little is known of its fate in the body. That it readily penetrates all body tissues has been assumed on the basis of its therapeutic efficacy, but has never been demonstrated. In order to provide experimental verification of this assumption and to gain some information on its intermediate metabolism, the concentrations of sulfanilyl-2-aminopyridine in the tissues and fluids of the dog have been determined at different intervals after administration of varying doses.

EXPERIMENTAL

Two-tenths gram of sulfanilyl-2-aminopyridine† per kilogram body weight was suspended in approximately 25 to 30 c.c. of water and partially dissolved by the addition of several drops of concentrated hydrochloric acid. This solution was then administered by stomach tube to dogs under nembutal anesthesia. At specified time intervals from fifteen minutes to twenty-four hours, aqueous humor, blood, and cerebrospinal fluid were removed. The blood was obtained from the external jugular or femoral veins and the cerebrospinal fluid by suboccipital puncture. Only one sample of cerebrospinal fluid and two samples of aqueous humor could be obtained from each dog since the composition of these fluids is known to change after paracentesis.[1] At the conclusion of the experiment, the dog was killed by intravenous injection of ether, and portions of the various tissues were removed as rapidly as possible for analysis. Representative samples of approximately 1 Gm. were weighed and transferred to mortars, where they were thoroughly ground with sand and a minimum volume of 3 per cent trichloracetic acid. This mixture was transferred quantitatively to a 15 c.c. centrifuge tube and allowed to stand overnight in the icebox. After centrifugation the clear supernatant fluid was decanted into test tubes calibrated at 10 c.c., and the precipitate twice washed with 2 c.c. portions of the trichloracetic acid. The supernatant liquid and washings were diluted to the mark with more acid. This solution was diazotized and coupled with alpha dimethylnaphthylamine according to the method of Marshall and Litchfield,[2] observing the precautions of Stevens and Hughes.[3] When the solution was not perfectly clear, the turbidity was removed by filtration. Photometric‡ readings were

*From the Departments of Chemistry and Ophthalmology, Northwestern University Medical School, Chicago.
Received for publication, July 31, 1939.
†Generously supplied by Merck and Co., Inc., Rahway, N. Y.
‡Cenco—Sheard-Sanford "Photelometer," using green filter No. 2.

then made to determine the concentration of sulfanilyl-2-aminopyridine. When plotted on semilogarithmic paper, the readings were found to be a straight line function of the concentration for all solutions containing less than 0.060 mg. Solutions more concentrated than this should be diluted to fall within the range. Blood was analyzed as described by Marshall and Litchfield,[2] except that 5 volumes of 15 per cent trichloracetic acid were employed instead of 4 volumes advocated in their method. This slight alteration gave us water-clear filtrates, whereas with 4 volumes, the filtrates were faintly colored. The volume of saponin employed was correspondingly decreased. Aqueous humor and cerebrospinal fluid were similarly analyzed.

RESULTS

As can be seen from Table I, sulfanilyl-2-aminopyridine has been found in

TABLE I

SULFANILYL-2-AMINOPYRIDINE CONCENTRATIONS* AFTER LARGE AND THERAPEUTIC DOSES

TIME AFTER ADMINISTRATION	WITH LARGE DOSES									WITH THERAPEUTIC DOSES
	MINUTES		HOURS							
	15	30	1	2	3	4	6	12	24	24
Blood	0.4	1.5	3.8	5.1	6.2	6.1	5.4	4.7	3.1	2.5
Spinal fluid		0.2	2.2	2.1	3.5	3.9	4.1	5.3	2.8	1.7
Aqueous humor	0.2	0.4	1.6	2.9	4.1	5.8	4.9	4.4	2.7	1.5
Vitreous humor		0.6	0.6	1.6	2.1	3.2	1.8	4.0	2.2	0.2
Sclera		0.7	2.4	5.0	6.2	7.0	4.1	11.5	2.9	0.8
Cornea		1.9	2.0	4.1	5.6	6.8	4.3	7.7	3.9	0.3
Lens		0.2	0.2	0.5	0.6	1.1	0.6	4.4	2.1	<0.1
Retina		0.6	5.7	6.3	4.6	6.3	4.4	11.5	3.4	1.8
Tendon			0.8	1.3		6.0	4.1	3.3	3.6	1.0
Bone marrow			0.9	1.3		6.1	4.9	4.2	2.5	0.2
Heart			1.6	2.9		5.3	2.7	2.7	2.0	0.1
Skin			0.8	3.1		6.8	3.9	8.7	4.6	1.4
Liver			4.7	4.9		9.2	4.1	10.7	2.4	0.9
Muscle			2.1	3.3		7.2	3.1	6.9	3.9	0.4
Fat			1.1	1.1		1.8	2.3	3.3	1.6	0.6
Lymph gland				5.8				4.5		
Spleen			3.5	4.5		6.6	4.1	7.1	3.0	0.8
Gastric wall			0.5	2.0		8.5	4.3	5.7	3.7	0.3
Gastric mucosa			17.9	30.9		27.3	45.5	12.9	2.9	0.8
Intestinal wall			3.1	1.6		7.0	5.1	6.7	3.9	0.3
Intestinal mucosa			5.9	1.3		6.0	2.4	2.1	5.0	1.3
Kidney			2.8	8.0		8.8	7.1	12.3	3.9	0.4
Pancreas			2.0	1.6		5.2	5.1	8.1	0.4	0.2
Adrenals			3.9	2.3		7.1	5.2	4.9	2.3	0.9
Brain				1.8		3.5	2.1	2.8	2.6	<0.1
Nerves			2.4	2.7		9.0	6.3	6.1	3.2	1.1
Lung			2.1	4.7		5.3	7.3	6.1	3.9	0.1
Trachea			1.0			6.1		2.8	1.8	
Thyroid			3.8	2.0		11.3	5.1	4.8	3.8	<0.1

*Blood, cerebrospinal fluid, and aqueous humor are expressed as mg. per 100 c.c. All others are expressed as mg. per 100 Gm. moist tissue.

every tissue or fluid thus far analyzed. It was found in the blood within fifteen minutes after administration and attained its greatest concentration between the third and fourth hours. In the aqueous humor the values were somewhat lower, but the shape of the curve was similar to that of blood. However, the

penetration into the cerebrospinal fluid appeared to be somewhat slower since the maximum value was secured at the end of twelve hours, at which time it was actually higher than that of the blood. The lens showed the slowest rise of any tissue analyzed, an observation previously reported with sulfanilamide.[4] At the end of six hours, it contained only 0.6 mg. sulfanilyl-2-aminopyridine per 100 Gm. of lens. Most of the tissues followed the curve for blood and aqueous humor, attaining their highest concentration about the fourth hour. A number, however, did not reach a peak until the sixth to the twelfth hour. The concentration in the gastric mucosa was several times that of any other tissue. This was probably due to the mode of administration. Of the other tissues, the kidney, retina, sclera, and thyroid showed the largest amount of sulfanilyl-2-aminopyridine, and fat and the lipid rich brain contained the least.

In the experiment just described sulfanilyl-2-aminopyridine was employed in doses considerably higher than those recommended for clinical use. The effect of the drug administered in therapeutic doses was next investigated.

Dogs were placed under nembutal anesthesia, and 30 mg. sulfanilyl-2-aminopyridine per kilogram body weight were given by stomach tube. An additional 15 mg. per kilogram were administered every four hours for the duration of the experiment. Blood was removed after one and two hours and at the end of each four-hour period. Aqueous humor was aspired after twelve and twenty-four hours, and cerebrospinal fluid and the various tissues were analyzed after twenty-four hours. The analyses were performed in the manner previously described.

The blood concentration remained quite constant for the period examined, ranging from 1.1 to 2.5 mg. per 100 c.c. from the second to the twenty-fourth hour. The aqueous humor and the cerebrospinal fluid contained slightly lower amounts (1.1 to 1.7 mg. per 100 c.c.) for this period. The values for the tissues are listed in Table I and range from a trace to 1.8 mg. per 100 Gm. of tissues.

DISCUSSION

It has already been shown[4, 5] that sulfanilamide readily penetrates into all tissues thus far examined. This fact has now been demonstrated for sulfanilyl-2-aminopyridine. As would be expected from its larger size, its penetrability into the cell is somewhat lower than that of sulfanilamide. Thus, the blood of dogs receiving 0.20 Gm. sulfanilamide per kilogram body weight contained 18.0 mg. per cent after four hours, whereas dogs receiving similar doses of sulfanilyl-2-aminopyridine contained only 6.1 mg. per cent. A similar diminution was noted in the other fluids and tissues analyzed. Despite this difference in the absolute content, the shape of the curves was very similar for both compounds. A greater variability was noted in the concentration of sulfanilyl-2-aminopyridine than sulfanilamide. Other workers[6, 7] have already called attention to this fact.

Considerably lower values were obtained when therapeutic doses were administered. These values were also lower than those secured on human beings receiving a proportional dose. Thus, the blood sulfanilyl-2-aminopyridine in our patients averaged 1.7 mg. per cent, whereas with patients similarly treated, the blood level was about 4 mg. per cent.[8] The blood sulfanilamide in dogs

receiving therapeutic doses has also been shown to be considerably lower than those obtained on man.[4] It seems probable that the amount of sulfanilyl-2-aminopyridine per unit weight required to maintain a definite blood level increases as the animal becomes smaller. However, it must be remembered that the superiority of a high blood concentration has not been definitely established. Thus Flippen and co-workers[9] found great variation in the concentration of sulfanilyl-2-aminopyridine among individuals receiving the same dosage, with no marked difference in chemotherapeutic effect. Further work is necessary to relate fluid and tissue concentrations with therapeutic efficacy.

CONCLUSIONS

1. The method of Marshall and Litchfield for sulfanilamide determination has been modified to allow sulfanilyl-2-aminopyridine estimation in tissues.

2. The concentration of sulfanilyl-2-aminopyridine in various tissues and fluids of the dog has been determined one, two, three, four, six, twelve, and twenty-four hours after administration of 0.2 Gm. per kilogram body weight. The maximum concentration is reached about the fourth to the sixth hour.

3. The level in the blood and aqueous humor was determined when therapeutic dosage was employed.

The authors wish to express their appreciation to Professors C. J. Farmer and S. Gifford for the suggestions and criticism so freely given during the progress of this work.

REFERENCES

1. Duke-Elder, W. S.: The Nature of the Intra-Ocular Fluids, Brit. J. Ophth. Suppl. III, 1927.
2. Marshall, E. K., Jr., and Litchfield, J. T.: The Determination of Sulfanilamide, Science **88**: 85, 1938.
3. Stevens, A. N., and Hughes, E. J.: Para-Aminobenzenesulfonamide: Notes on the Colorimetric Assay, J. Am. Pharm. A. **27**: 36, 1938.
4. Bellows, J. G., and Chinn, H.: The Distribution of Sulfanilamide in the Eye, J. A. M. A. **112**: 2023, 1939.
5. Marshall, E. K., Jr., Emerson, J., Jr., and Cutting, W. C.: The Distribution of Sulfanilamide in the Organism, J. Pharmacol. & Exper. Therap. **61**: 196, 1937.
6. Long, P. H., Bliss, E. A., and Feinstone, W. H.: The Effects of Sulfa-Pyridine, Sulfanilamide, and Related Compounds in Bacterial Infections, Pennsylvania M. J. **42**: 483, 1939.
7. Marshall, E. K., Jr., Bratton, A. C., and Litchfield, J. T., Jr.: The Toxicity and Absorption of 2-Sulfanilamidopyridine and Its Soluble Sodium Salt, Science **88**: 597, 1938.
8. Stokinger, H. E.: Absorption, Acetylation and Excretion of 2-Sulfanilamido Pyridine (Dagenan, M. & B. 693), Proc. Soc. Exper. Biol. & Med. **40**: 61, 1939.
9. Flippen, H. F., Lockwood, J. S., Pepper, D. S., and Schwartz, L.: Treatment of Pneumococcic Pneumonia With Sulfapyridine. Progress Report on Observations in 100 Cases, J. A. M. A. **112**: 529, 1939.

LABORATORY METHODS

I. STUDIES OF LIVER FUNCTION IN HEALTH AND DISEASE*

Observations on a Simple and Accurate Method for Quantitative Determination of the Bile Salts in Urine and Bile

Lester M. Morrison, M.D., and William A. Swalm, M.D., Philadelphia, Pa.

IT IS NOW one hundred and fifty years since the first attempts of Foureroy and Vauquelin (1790) and later of Thenard (1807) and Berzelius (1809) to identify the various constituents of bile. Since that time there has not been a satisfactory method for the clinician in the practice of medicine to utilize the facts unearthed by innumerable investigators relating to the function of the liver in health and disease.

This communication presents the results of three years' studies in over 4,000 urine specimens and over 5,000 bile specimens on more than 500 patients over extended periods of time.

The method herein described is a stalagmometric one of estimating the surface tension of bile and urine, which gives the exact quantity of bile salts present. To date other tests for this purpose have presented certain noteworthy disadvantages.

(a) They have been elaborate, requiring the services of a skilled chemist, as with Doubilet's technique.

(b) They have been nonspecific for bile salts, as with the Pettenkoefer reaction, originating in 1848 and still in use under numerous modifications. The Pettenkoefer reaction has been shown to react positively with as many as forty other substances, by Thudicum (1868), Mylius (1887), Udranszky (1887), Tashiro and Mills, and Walker.

(c) Numerous tests, as those proposed by Chabrol, Charonnat, Cottet and Blonde, by Goiffon, Nepveux and Chaleil, Peoples, Etchaverry, Nakagawa and Fujikawa, Nakagawa and Yoshikawa, Sullman and Schaub, Borgatti (Giordano and Levi modification), and others, are unsatisfactory for clinical use due to uncertainty of color reactions, elaborate technique, great time consumption, expensive apparatus, or a combination of some of these impractical procedures. As an example, Minibeck's technique requires an elaborate "fluorescent microscopic spectrophotometer." The price of this instrument ranges in the neighborhood of $800 to $1000, and in addition must be designed and built individually.

(d) The Gregory and Pascoe reaction has been used extensively in recent years, depending upon a colorimetric reaction. However, as pointed out by Sobotka and especially by Doubilet, "it is specific for cholic acid alone and is

*From the Department of Medicine, Temple University Medical School, Philadelphia.

Grateful acknowledgment is expressed to Dr. Robert H. Hamilton, Jr., for invaluable biochemical aid and to Marion Fountain for technical assistance.

of little value in the analysis of human bile which contains a large proportion of desoxycholic acid." At the maximum, cholic acid comprises 50 per cent of the total, as has been estimated by Doubilet and Colp, Sobotka, Weiland and Revery, and others, in human fistula bile from persons having had common duct obstruction. When inflammation of the ducts was noted at operation, Doubilet and Colp found cholic acid to comprise only one-sixth of the total bile acids. The error in relying on this variable cholic acid index as to the total amount of bile acids by the Gregory and Pascoe method is further seen when one finds that in addition to cholic acid, the other human bile acids are oxalic acid (Larvonat); desoxycholic acid, anthropodesoxycholic acid, lithocholic acid, chenodesoxycholic acid (Weiland); cholalic acid (Schotten); fellic acid (Schotten); cholic acid and taurochenodesoxycholic acid (Weiland); and glycodesoxycholic acid (Hammarsten). Sobotka has likewise emphasized the wide variations cholic acid undergoes and its ratio to the other bile acids as soon as pathology sets in.

The surface tension method of estimating bile acids or salts in the urine has been investigated by Schemensky in 1920, and by Doumers, Gilbert, Chabrol, Benard, Mueller, Adlersberg, Meyer, Eppinger and Ranzi, Duco and Panza, and the essayists.

The Doumers first established the fact already known that bile salt solutions in distilled water in increasing concentrations lowered their surface tension following a regular and symmetrical curve to a certain limit (Doumer's law).

However, Duco and Panza found that this law is not applicable to urine or bile since electrolytes, as sodium or potassium salts, may cause considerable variations in the bile salt estimations (Schulze-Hardy law). This error was avoided by Duco and Panza's introduction of 1 per cent phosphoric acid, sufficient to bring the solution to the isoelectric point of pH^2. At this isoelectric point there is no alteration in bile salt solution by extraneous electrolytes or colloids. Hence urine or bile at a pH^2 gives a surface tension figure which is specific for bile salts alone. When the solutions are at a dilution of less than 1:1,000, charcoal adsorbs practically 100 per cent of the bile salts. If then the surface tension of the bile or urine is taken at pH^2, and again taken after extractions with medicinal animal charcoal, the difference is accurately expressed in quantity of bile salts. Comparison is then made with a graph which gives the surface tension of increasing concentrations of pure sodium glycocholate in distilled water. This is shown for urine and bile in Figs. 1 and 2 in the stalagmometer used.

An ordinary stalagmometer is used to determine the number of drops of bile or urine by a hand counter.

We present a modified technique in lieu of that proposed by the foregoing investigators studying surface tension, and most recently by Duco and Panza, giving a simplified test with greater accuracy for clinical purposes. These modifications are as follows: (1) Considerable time is saved by omitting the extraction by medicinal animal charcoal. We found that if an accurate isoelectric point of pH^2 in the bile or urine were obtained, for clinical purposes practically 100 per cent of the bile acids are obtained directly from the

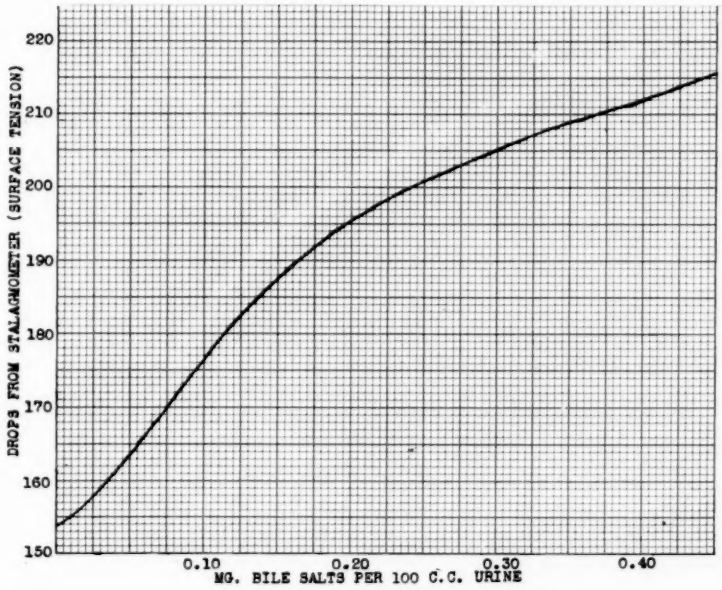


Fig. 1.

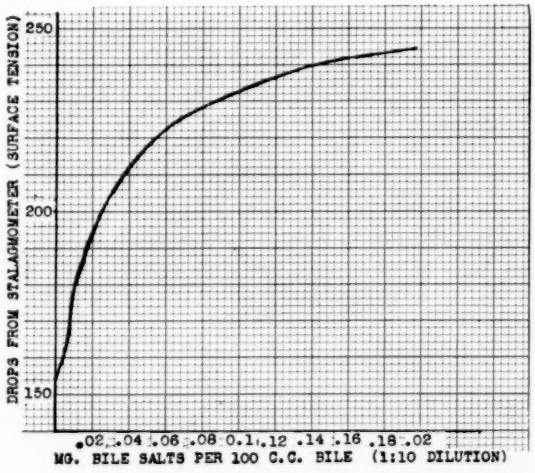


Fig. 2.

stalagmometer. Hence the charcoal treatment of the solution is unnecessary and time-consuming. (2) Instead of preparing a special solution of diluted phosphoric acid, concentrated hydrochloric acid is used for greater convenience and practicability. (3) The modification recommended by Dueo and Panza, of arbitrarily adding 3 drops of the acid solution to the urine or bile to get the isoelectric point, is frequently inaccurate, particularly so in the urines. It is easily demonstrated that some urines or biles require several times this amount to get at the isoelectric point of pH[2]. If this is not secured, the calculations, as advised by Dueo and Panza themselves, may be very inaccurate, due to the variable contents of electrolytes and colloids occurring in different specimens examined. The pH of various specimens from the same individual is found to undergo wide variations at differing intervals.

The only accurate and dependable way to insure a pH[2] is to employ an indicator for this desired acidity. Tropeolin 00, as suggested by Dr. Robert Hamilton, is the best and most practical indicator for this desired pH. Hence a piece of filter paper dipped in the tropeolin 00 solution will turn pinkish red when the bile or urine being tested is at the correct pH[2].

The surface tension of the urines and biles should be taken at about room temperature and at a rate of approximately 30 drops per minute. A stalagmometer can easily be made from a 15 c.c. pipette, by fusing a small-lumened glass to its end. The cost is approximately $0.50 to $1.00. A standardized stalagmometer can be bought from any appropriate chemical supply concern for a relatively small sum.*

There are no normal or pathologic standards reported in the literature of the bile salt concentration in urine. Previous investigators have reported bile salt concentrations in the bile by methods described as inaccurate or impractical for clinical purposes. Hence in a separate communication these standards for bile and urine are established. It will be shown that the bile salt concentration in the bile is the most sensitive, direct, and accurate method of determining the physiologic and pathologic function of the liver. The value of bile salt estimations in the urine will also be reported.

SUMMARY

1. Observations on a simple, sensitive, and reliable method of determining the bile salt concentration in urine and bile are presented.

2. The bile salt concentration is determined by a surface tension method (stalagmometric).

3. The method requires no training or skill, and entails very little expense and time.

4. This method is most practical and accurate for determining the clinical significance of variations in the bile salt concentration of bile and urine. Clinical studies based on this technique follow in a subsequent report.

REFERENCES

Adlersberg, David: Auscheidung oberfl. aktiver Stoffe i. Harn. L. exp. Med., Ztschr. f. d. ges. exper. med. **42**: 194-212, 1924.

Berzelius, J.: Composition des fluides animaux, Ann. Chem. **71**: 218-224, 1809. Ibid. **88**: 26-74, 113-145, especially 119, 1815.

Borgatti, Guiseppe: Bull. d. Soc. ital. di biol. sper. **7**: 804, 1932.

Chabrol, E., Charonnat, R., Cottet, J., and Blonde, P.: New Technique for the Detection of Bile Salts, Compt. rend. Soc. de biol. **115**: 834, 1934.

Coquelet, O.: Reaction Furfural, Compt. rend. Soc. de biol. **97**: 747-750, 1815-1818, 1927.

Doubilet, Henry: Differential Quantitative Analysis of Bile Acids in Bile and in Duodenal Drainage, J. Biol. Chem. **114**: 288-308, 1936.

Doubilet, H., and Colp, R.: Differential Analysis of Bile Acids in Human Bile From Fistulas, Arch. Surg. **34**: 151-173, 1937.

Doumer, E., and Doumer, E.: La mesure du taux des substances, qui abaissant la tension superficielle de l'urine, Compt. rend. Soc. de biol. **85**: 177, 1921.

Doumer, E.: L'action du taurocholate de sounde sur la tension superficielle de l'eau, Compt. rend. Soc. de biol. **85**: 1138, 1921.

Duco, C. L., and Panza, P. T.: Los ácidos biliares nuevo método de dosaje. Colaluria fisiológica, Semana méd. **1**: 1193-1198, 1930.

Eppinger, M., and Ranzi, E.: Die hepato-lien en Erkrankungen, in Enzyklop. d. klin. Med. Berlin, 1920, J. Springer.

Etcheverry, M. A.: Arch. argent. de enferm. d. ap. digest. y. de la nutrición **7**: 193, 1932.

*This can also be purchased from the Precision Thermometer and Instrument Co., Broad and Brandywine Streets, Philadelphia.

Fourcroy, A. F., and Vauquelin, L. N.: Copie de quelques dicouvertes chimiques, Ann. de Chem. **6**: 177-182, 1790.
Gilbert, A., Chabrol, E., and Benard, H.: Recherches stalagmometriques sur le NaCl, Compt. rend. Soc. de biol. **84**: 65-67, 1921.
Goiffon, R., Nepveux, F., and Chaleil: Compt. rend. Soc. de biol. **121**: 425-427, 1936.
Gregory, R., and Pascoe, I. A.: Quantitative Determination of Bile Acids, J. Biol. Chem. **83**: 35-42, 1929.
Herzfeld, E., and Haemmerli, A.: Schweiz. med. Wchnschr. **54**: 141-145, 1924; Ibid. **55**: 142-149, 164-172, 1925.
Meyer, E. C.: Bestimmung d'Gallensauren un Duodenalsaft, Biochem. Ztschr. **140**: 356-367, 1923; Klin. Wchnschr. **2**: 1317, 1923.
Idem: Nachiveis und Schaetzung d'Gallensauren im Urin, Deutsches Arch. f. klin. Med. **147**: 274-286, 1925.
Minibeck, H.: Beitrage zur quantitativen fluoreszenzphotometrischen Mikroanalyse; fluoreszenzphotometrische Bestimmung der Gallensäuren in Duodenalsaft, Fäzes und Organen, Biochem. Ztschr. **297**: 214-220, 1938.
Mueller, H., Jr.: Klin. u. Path. Schweiz. Med. Wo. **52**: 110, 1922.
Mylius, F.: Zur Kenntnis der Pettenkofer'schen Gallensaureraction, Ztschr. f. physiol. Chem. **11**: 492-496, 1887.
Nakagawa, S., and Yoshikawa, K.: J. Biochem. **13**: 321-341, 1931.
Nakagawa, S., and Fujikawa, H.: J. Biochem. **12**: 399, 1930.
Peoples, S. A.: Proc. Soc. Exper. Biol. & Med. **30**: 1117, 1933.
Schemensky, W.: Biol. **105**: 229-252, 1920.
Sobotka, H.: Physiological Chemistry of the Bile, Baltimore, 1937, Williams & Wilkins Co.
Sullman, H., and Schaub, L.: Biochem. Ztschr. **251**: 369, 1932.
Tashiro, S., and Mills, C. A.: Note on the Antagonistic Action of Cholesterol to the Bile Salts in Blood Coagulation, Med. Bull. Univ. Cincinnati **6**: 98-99, 1931.
Thenard, L. J.: Deux memoires sur la bile, Ann. de chim. **64**: 103-112, 1807.
Thudicum, J. L. W.: Tenth Report of the Medical Officer of the Privy Council, London **17**: 243, 1868.
Udransky, L.: Ueber die Bezeihung, im Harne bereits vorgebildeten, oder daraus durch einfache Proceduren clarstellbaren, Farbstaffe zu den Huminsubstanzen, Ztschr. f. physiol. Chem. Strassburg **11**: 537-560, 1887.
Walker, E.: The Alleged Presence of Bile Salts in Normal Blood, Biochem. J. **24**: 1489-1492, 1930.
Weiland, H., and Revery, G.: Gallensaeuren xxi. L. Kenntn. d. menschl. Galle I, Hoppe Seyler's Ztschr. f. physiol. Chem. **140**: 186-202, 1924.

THE FUCHSIN-FORMALDEHYDE METHOD OF STAINING ACID-FAST BACILLI IN PARAFFIN SECTIONS*

G. L. Fite, M.D., Honolulu, T. H.

THE fuchsin-formaldehyde method, briefly mentioned in a previous discussion of the conditions affecting the staining of acid-fast bacilli,[1] has been used routinely in this laboratory for a year and a half, having been found to be the most reliable means of staining acid-fast bacilli in sections permanently, irrespective of the fixative employed. It has been found, however, that its usefulness depends upon the quality of the formaldehyde solution used.

The protocol of the method is as follows: washing in tap water after each step being understood:

(1) Stain in

New fuchsin	0.5 Gm.
Phenol crystals	5.0 Gm.
Alcohol, methyl or ethyl	10.0 c.c.
Distilled water, to make	100.0 c.c.

*From the Division of Infectious Diseases, Leprosy Investigations, U. S. Public Health Service, Honolulu.
Published with the permission of the Surgeon-General, U. S. Public Health Service.
Received for publication, June 26, 1939.

at 60° C. (paraffin oven) overnight for from twelve to twenty-four hours, or at room temperature for from twenty-four to forty-eight hours. The longer periods are recommended for *M. leprae*.

(2) Freshly distilled aqueous formaldehyde, 5 to 30 per cent, five minutes.

(3) Hydrochloric acid, 2 per cent, in 95 per cent alcohol, ten minutes.

(4) Potassium permanganate, 1 per cent aqueous, until brown, usually two to five minutes.

(5) Oxalic acid, 2 per cent, one minute.

(6) Harris' hematoxylin, two minutes.

(7) Van Gieson's stain, 3 minutes.

Acid fuchsin	0.1 Gm.
Picric acid	0.5 Gm.
Distilled water, to make	100.0 c.c.

(8) Without washing, dehydrate in alcohol, clear in xylol, and mount in balsam.

Nuclei stain brown, connective tissue fibers red, muscle fibers yellow, and acid-fast bacilli dark ultramarine blue.

DISCUSSION

The preferred fixative is a mixture of equal parts of 10 per cent formaldehyde and 95 per cent ethyl alcohol. Fixation for several days, three to five or more, is noticeably superior to brief, twenty-four-hour fixation. Tissues preserved in this medium have given excellent results after preservation for several months.

The use of formaldehyde of the highest purity in the staining process is important. Aged or exposed solutions often give poor or no results, decolorizing the bacilli. The "reagent" grade of formaldehyde, preserved in dark bottles, usually is satisfactory, but any commercial formaldehyde, distilled through a simple water-cooled glass condenser, gives a usable product containing about 30 per cent formaldehyde. Solutions a few days old are satisfactory, but after three or four weeks, they may become very unreliable. It is advisable to use freshly distilled formaldehyde and to renew it for each batch of slides. Some dye is extracted by the formaldehyde, and the section is rendered a deep blue color.

The acid alcohol outwardly does very little, but seems to facilitate the subsequent bleaching process. The bacilli, rendered blue by the formaldehyde, will withstand vigorous treatment without being decolorized. The previous soaking in formaldehyde prevents much shrinkage which might otherwise take place during the bleaching. Tissues fixed without the use of heavy metals are readily totally decolorized, but a moderate residual pale blue color is largely masked by the hematoxylin.

By this method lepra bacilli, at times impossible to stain in paraffin sections by other means, have been found to be readily colored. The dark blue bacilli lend themselves very well to photomicrography, with purposeful understaining of the tissues.

REFERENCE

1. Fite, G. L.: Staining of Acid-Fast Bacilli in Paraffin Sections, Am. J. Path. **14:** 491, 1938.

A RAPID MEANS OF OBTAINING MANGANESE-FREE IRON*

THOMAS W. RAY, PH.D., MILWAUKEE, WIS.

MANGANESE-FREE iron is necessary in certain feeding experiments where manganese is to be excluded from the diet and where iron must be added. No iron salt or iron wire which we have tested is free from manganese, and there has, heretofore, been no rapid and convenient means of separating the two elements.

Reitzenstein[1] found that when finely pulverized ferrous chloride was vigorously shaken with pyridine and the mixture heated, a canary-colored crystalline mass appeared, which upon recrystallization from pyridine had the formula $FeCl_2.3C_5H_5N.2H_2O$. This compound was unstable and rapidly changed to a basic salt. Pfeiffer[2] prepared an iron pyridine compound which he believed to be identical with that of Reitzenstein's, and which when exposed to the air, turned brown. Later, Moore and Miller[3] found that if pyridine is added to a solution of ferric chloride containing free hydrochloric acid, iron is precipitated quantitatively as the hydroxide. In all probability the brown-colored compound obtained by Pfeiffer was iron hydroxide. Moore and Miller also found that when pyridine is added to a neutral solution of a manganese salt in the cold, no precipitation of manganese takes place, but upon heating oxidation occurs and manganese comes down. They noted, however, that if the solution is made acid with hydrochloric acid, heat could then be applied without the precipitation of manganese. These observations furnished them with an effective means of separating the two elements. They precipitated ferric hydroxide by pyridine from a solution of ferric chloride containing hydrochloric acid, removed the solution by filtration, and washed the precipitate with water containing pyridine. The ferric hydroxide was dissolved in hydrochloric acid and reprecipitated by pyridine. After a number of reprecipitations they obtained manganese-free ferric hydroxide.

Orent and McCollum[4] prepared manganese-free iron by a modification of the Moore and Miller procedure, using filtration, in which they reprecipitated the iron as the hydroxide by pyridine eight times, and finally obtained a product containing less than 0.5 p. p. m.

By a further modification of the Moore and Miller procedure in which a special means of precipitation is used and in which centrifugation rather than filtration is resorted to, we have been able to prepare manganese-free ferric hydroxide from a solution of ferric nitrate containing 0.002 per cent manganese with one precipitation by pyridine, in which no trace of manganese can be detected. Iron wire containing as much as 0.2 per cent manganese,

*From the Department of Biochemistry, Marquette University Medical School, Milwaukee. Received for publication, July 11, 1939.

or ordinary ferric nitrate, ferric chloride, or ferric sulfate can be freed from manganese by this procedure with two precipitations.

PROCEDURE

Four grams of ferric nitrate are dissolved in 500 c.c. of water in a large beaker. To this solution, containing no hydrochloric acid, 7.5 c.c. of pyridine are dropped in from a 10 c.c. burette, a small drop at a time, while the contents of the beaker is rapidly revolved. Since the pyridine is added slowly and the solution thoroughly mixed, supersaturation takes place simultaneously throughout the entire volume of liquid, causing the hydroxide to appear in exceedingly small particles. This means of precipitation results in the bringing down, by occlusion, of a minimum amount of soluble manganese salt. An additional 0.5 c.c. of pyridine is run in and the hydroxide allowed to settle. The addition of more pyridine to the nearly colorless liquid causes no further precipitation. After removing most of the liquid by decantation, the contents of the beaker are transferred to two 50 c.c. centrifuge tubes. The tubes are rotated for not more than two minutes at a low rate of speed, after which as much of the remaining liquid as possible is poured off. The rotation must be slow and the time short, otherwise the hydroxide will pack, and packing makes washing ineffective. The precipitate, which is very soft and still contains a small amount of liquid, is washed into 500 c.c. of water containing 0.5 c.c. of pyridine. After vigorous rotation of the beaker to insure thorough mixing, the precipitate is allowed to settle and the water removed as previously. Five such washings are sufficient to free the hydroxide from manganese.

No hydrochloric acid is used in this procedure because precipitation of the hydroxide is brought about without heat, and manganese does not come down in the cold, even in the absence of hydrochloric acid. When a second precipitation is required, the residue is dissolved in hydrochloric acid, but just enough to bring it into solution. If acid is used in excess, more pyridine is required for precipitation.

SUMMARY

Removing soluble manganese salts, by filtration, from ferric hydroxide precipitated in the usual way is unsatisfactory. The precipitate brings down with it much manganese, and filtration causes the precipitate to form a gelatinous mass which makes washing slow and ineffective. Good results, however, can be accomplished by precipitating as already described, and by centrifugation. By regulating the time and the rate of speed of the tubes the washings can be removed by decantation, and the hydroxide kept in its original finely divided form. This makes for effective washing.

CONCLUSIONS

A procedure is described for removing soluble manganese salts from iron by a special means of precipitation, and by removing the washings by centrifugation rather than by filtration.

REFERENCES

1. Reitzenstein, Fritz: Einwirkung von Pyridin auf festes Ferrochlorür, Z. anorg. Chem. **18:** 283, 1898.
2. Pfeiffer, P.: Eisenchlorürpyridin, Z. anorg. Chem. **29:** 138, 1901.
3. Moore, Richard B., and Miller, Ivy: Separation of Iron From Manganese, J. Am. Chem. Soc. **30:** 593, 1908.
4. Orent, Elsa R., and McCollum, E. V.: Effects of Deprivation of Manganese in the Rat, J. Biol. Chem. **92:** 662, 1931.

A NOTE ON THE PREPARATION OF HEMATOPORPHYRIN*

ROBERT D. BARNARD, CHICAGO, ILL.

WITH the introduction of sulfanilamide and the occasional serious reaction that follows its administration, the detection of porphyrinuria, which sometimes accompanies these reactions, may become a procedure of routine laboratory analysis. Most of the methods for the detection of porphyrins are elaborate and require a familiarity with spectrophotometry.

In this laboratory it has been found possible to detect hematoporphyrin concentrations in aqueous solutions of one part in one million by examination for the brilliant red fluorescence of such solutions in "dark light" (the ultraviolet radiation of a water-cooled quartz Kromayer lamp from which visible light is filtered by a cobalt quartz screen). Before this procedure can be made available for general study, it will probably be necessary for hematoporphyrin to be available for the purpose of standardization. The material is not on the market in the pure form and even if it were, there would be some question as to whether the ordinary preparations represent true hematoporphyrin.

The majority of the methods for the preparation of hematoporphyrin from blood pigment depend on the use of heat and strong acids. It is now recognized, since the synthesis of porphyrins by Hans Fisher (1926) that many of the preparations of hematoporphyrin, by the older methods, are denaturated, a fact reflected in the variability in absorption spectrum and solubility of different specimens.

The method of Laidlaw (1904) in which the iron is removed from ferroheme (reduced hematin) by the action of weak acid in the cold, is free from these objections. It has been found to be eminently satisfactory, gives an easily reduplicable product, and would seem to yield a hematoporphyrin that is close in its chemical structure to the nucleus as it exists in hemoglobin. In the original method the hemoglobin is kept in a state of reduction by treatment with hydrazine hydroxide and treated with 15 per cent hydrochloric acid which dissociates the pigment into globin and ferroheme, and then splits the iron from the latter. The final product is contaminated with a little protein that is difficult to remove. For this reason, the method has been modified by

*From the Chemical Laboratory of the Northern Illinois Eye Clinic, Chicago.
Received for publication, July 18, 1939.

(1) precipitation of the corpuscular cholesterides, (2) digestion of the protein with pepsin, and (3) chloroform extraction of the porphyrin. Reduction is carried out by suction evacuation, which is continued throughout the process, since it has been found that in acid solution the reducing action of hydrazine is not manifest.

The yield by the present method is practically 100 per cent, though, since there are no expensive reagents, like hydrazine hydroxide, used, this factor would hardly enter into consideration as a reason for the adoption of the method.

PROCEDURE

Five hundred cubic centimeters of fresh, citrated, oxalated, or defibrinated blood (that of the dog, cow, pig, and human being has been found satisfactory) are allowed to stand in a 1 liter pyrex Erlenmeyer suction flask until the corpuscles have settled and the serum is removed with a suction pipette. One gram of U.S.P. pepsin is added, and after it has dissolved, 50 c.c. of ethyl ether and then 50 c.c. of toluene are added. The contents are shaken until they gelatinize. An excess of toluene may remain, but this should not be removed as it prevents frothing during the subsequent evacuation.

A one-holed rubber stopper which fits the neck of the flask snugly and through which has been placed the stem of a short, glass-stoppered separatory funnel so that the outlet is at least 3 inches from the fluid level when the stopper is inserted, is prepared. The side arm outlet of the flask is connected through a short length of rubber tubing, carrying a heavy screw clamp, to a T-tube which is connected with a vacuum pump. (A small water-trap type of pump is sufficient.) The free end of the T-tube is fitted with a rubber tube and pinch clamp.

With the stopcock of the separatory funnel closed and the glass stopper in place, suction is applied for one hour. Usually the reduction to hemoglobin will occur much sooner, but the suction is maintained to remove all free oxygen and ether, not only during the addition of the acid, but also for a considerable time thereafter.

About 100 c.c. of concentrated hydrochloric acid are measured into the funnel, and the glass stopper is replaced. The stopcock is turned cautiously and the acid is admitted to the flask, about 5 c.c. at a time, with constant gentle agitation. It will be noticed that wherever the acid comes into contact with the corpuscle paste, a thick greenish-brown rubbery coagulum is formed. From 70 to 90 c.c. of acid are required for complete coagulation. The glass stopper is left in place during the addition of the acid; it prevents its spilling during agitation of the flask. Should the stopcock be inadvertently opened too widely and all the acid aspirated, the preparation will not be ruined by the advent of air, which admitted at this stage will result in the formation of acid hematin. The best results are obtained when just sufficient acid is admitted to cause complete precipitation of all protein.

The clamp on the tubing connected with the side arm is closed, the vacuum broken at the T-tube, and the flask disconnected and transferred to an incubator. In about three hours the material is a liquid sludge. This is divided

between each of two 250 c.c. centrifuge bottles until the dark brownish material is packed and the supernatant toluene can be decanted. The bottles are then inverted to drain.

About 75 c.c. of chloroform are added to each sample, and the sludge is comminuted in it with a glass rod. On centrifuging the chloroform settles to the bottom, the coagulum rising to the top as a grayish mass of claylike consistency. This is perforated with the glass rod and the chloroform solution is decanted. It is not necessary to filter the chloroform extract; in fact, this procedure is attended by considerable loss of the solution.

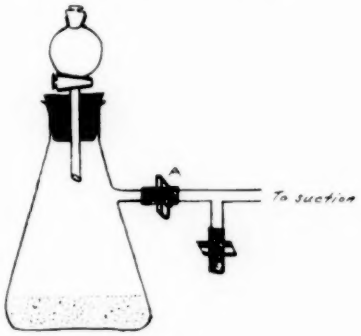


Fig. 1.

The chloroform solution from which the hematoporphyrin may be crystallized directly shows an intense red fluorescence as well as marked dichroism, being grass green by reflected light and purple red by transmitted light. On shaking with an equal volume of 2 per cent ammonium hydroxide, neutral amorphous hematoporphyrin is precipitated at the interface of the liquids. Stronger ammonia extracts the pigment from the chloroform as the ammonium salt, crystallizing as reddish brown rosettes on evaporation of the solution.

Neutralization of the ammonium hydroxide extract with dilute hydrochloric or acetic acid caused the precipitation of neutral hematoporphyrin. The latter was found to be so insoluble in water and in physiologic saline (less than 1:1,000,000 on the basis of the fluorescence test) that some question as to the reputed toxicity of *pure* hematoporphyrin arose. The same question has arisen repeatedly in connection with two other closely related substances, bilirubin and hematin, both of which are practically insoluble in the pure state at the pH of blood serum or tissue fluids, and for whose toxicity the weight of evidence appears to be in the negative. Insofar as ingestion by the water flea or contact with the human skin is concerned, the material prepared by the foregoing method seems to be devoid of toxic effects.

EXPERIMENTS WITH THE WATER FLEA

The technique described by Tinsley (1938) was followed. To a 500 c.c. culture of *Daphnia pulex,* 1 Gm. of amorphous hematoporphyrin was added. The animals survive indefinitely in the presence of the material. On microscopic examination the animals can be seen to continuously ingest and egest the pigment which passes through the entire alimentary tract without apparent

alteration. Examination of the culture water after twenty-four hours of such ingestion by a crowded culture of daphnia shows no solution of the pigment within the sensitivity of the fluorescence test.

EXPERIMENTS WITH HUMAN BEINGS

A 1 per cent solution of hematoporphyrin in acid alcohol was painted on an area 1 cm. square on the flexor surface of the forearm. After evaporation of the alcohol a brownish adherent film was left. This area was then irradiated with a carbon arc for ten minutes at a distance of 6 inches. Twenty-four hours later the pigment was removed with soap and water. The irradiated area, at this time, showed a moderate erythema except where protected by the hematoporphyrin film, where there had been no obvious change in the skin. Since this result indicated that hematoporphyrin could act as a screen against actinic rays, a film from a 1 per cent acid alcohol solution was painted over a small area of a lesion of psoriasiform lupus erythematosis on the forehead of a 40-year-old male and left in place for a week. There was no visible effect produced by this application as compared with similar unpainted areas in the same individual.

SUMMARY

A method for the preparation of hematoporphyrin is described. The material is practically insoluble in neutral aqueous solutions. It is not toxic by ingestion for *Daphnia pulex* and appears to be nonirritating to the human skin. Its solutions show a red fluorescence to ultraviolet light and the dried films act as a screen to that part of the spectrum which induces erythema of the skin following irradiation.

REFERENCES

Fisher, H.: Chlorophyll, Chem. Rev. **20**: 41, 1937.
Laidlaw, P. P.: Studies on Hematoporphyrin, J. Physiol. **31**: 465, 1904.
Tinsley, W. C.: Use of Daphnia in Study of Cathartic Action, J. Lab. & Clin. Med. **23**: 985, 1938.

NITRITE FERRIHEMOCHROMOGEN AS A REAGENT FOR REDUCING SUGARS*

ROBERT D. BARNARD, CHICAGO, ILL.

WHILE there are numerous specific chemical reactions whereby the presence of the reducing sugars may be detected and their quantities estimated, most of the routine laboratory reagents for this purpose depend upon the nonspecific reducing properties of these sugars toward various suitable oxidizing agents. Among these reagents in common use, we find in order of their frequency of utility, (1) alkaline cupric complexes, (2) ferricyanide solutions, (3) alkaline bismuth complexes, (4) alkaline picrate solutions, (5) alkaline silver or mercury complexes, and (6) solutions of redox potential indicator dyes, such as safranine, indigo, or methylene blue. All of these substances are capable of existing in a lower valence state, to which they may be brought by the action of any substance with a high reduction potential, such as dextrose at the boiling point, and in which state they exhibit physical or chemical differences from the oxidized form. In this sense, any of the reagents which depend upon the reducing powers of the sugar could hardly be made specific.

The alkaline metal complexes, however, have stood the test of time and laboratory utility. It is true that from a quantitative standpoint their reactions with the sugars have never been in true stoichiochemical relationship, but there are several excellent quantitative methods which have been standardized empirically and are being improved constantly. Most of the improvement in the copper reagents, particularly, are designed to prevent the reoxidation of the reduced form which is to some extent inhibited by the relatively insoluble form of the latter in the case of the metal complexes, whereas in the case of the redox indicator dyes, it is so rapid as to almost preclude their use. In fact, the only reagents that are free from this objection are the alkaline picrates and ferricyanides, the high oxidation potential of which prevents their reoxidation by atmospheric oxygen; at the same time this potential makes them easily reduced by substances other than dextrose to the effect that results of quantitative determinations for dextrose made on biological fluids with these reagents are invariably too high.

The ideal reagent in this category would have a low redox potential, but the reductant would be removed from participation in the redox system so that reoxidation could not occur. The possibilities for such a reagent appeared from a consideration of the recently elucidated properties of nitrite-ferrihemochromogen.

*From the Chemical Laboratory of the Northern Illinois Eye Clinic, Chicago.
Received for publication, July 18, 1939.

That "alkali hematin" solution was transformed to "reduce alkaline hematin" by heating with dextrose, has been known for over half a century. There seem to have been few attempts to utilize the reaction for the detection of dextrose because the reduced pigment rapidly reverts to the oxidized form. It later became recognized that pure hematin was irreducible by the reducing sugars in the absence of an adventitious "suitable" nitrogenous substance (hemochromogen former, Bertin-Sans and de Montessier, 1892) likewise present in its solution. It has been found that nitrite is such a hemochromogen former (Barnard, 1939) and that a solution of nitrite ferrihemochromogen, a greenish pigment, makes an excellent qualitative and semiquantitative reagent for reducing sugars, being converted by them to a scarlet pigment, nitric oxide ferrohemochromogen; the latter stable for several hours even in contact with atmospheric oxygen. The distinctiveness of the latter, as well as its characteristic absorption spectrum, which might make it applicable to a spectrophotometric method for the determination of these sugars, led to the development of a reagent which possesses several advantages over those commonly in use. The reagent is so flexible as to be equally applicable to the quantities of dextrose in the urine of a person with severe diabetes (4 per cent) or that in a 1:5 Folin-Wu filtrate of normal blood (0.02 per cent). It likewise is of quantitative significance, the time for reduction being in inverse proportion to the quantity of sugar present.

PREPARATION OF REAGENT

One hundred and sixty milligrams of crystalline ferriheme chloride (hemin*) are dissolved in 1 liter of 5 per cent tribasic sodium orthophosphate containing 1 Gm. sodium nitrite. The reagent is ready to use upon solution of its constituents and keeps indefinitely. When kept in soft glass containers, there may be a precipitation of silicates, but this does not affect its utility and may be prevented by keeping the reagent in hard glass containers.

Since hemin is a dibasic acid, the phosphate is incorporated to buffer the solution and also to precipitate the alkaline earths where the reagent is used for the detection and estimation of dextrose in urine, otherwise insoluble magnesium and calcium salts of hematin are formed.

The reagent was found to be unaffected by creatinine, chloroform, urates, or polyphenols. From this standpoint, its usage in urinalysis was obvious. The application to blood dextrose determinations will be the subject of a further communication.

DETECTION AND QUANTITATIVE ESTIMATION OF REDUCING SUGAR IN URINE

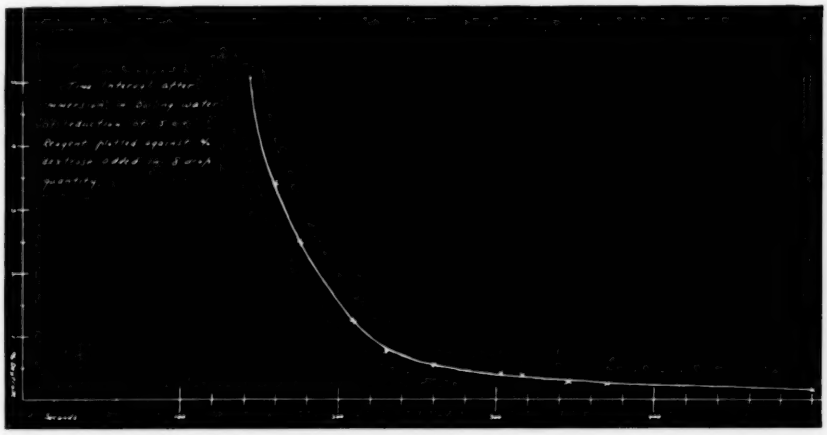
Five cubic centimeters of reagent and 8 drops of the urine to be tested are poured into an 8-inch by ⅝-inch test tube. The contents are mixed and the tube is immersed in a boiling water bath, which should be of sufficiently large volume that it continues to boil during the immersion. An evaporating dish of porcelain, resting on the bottom of a pyrex beaker, makes a suitable

*The hemin used in this study was prepared by the Pharmaceutical Division of the Armour Laboratories according to a modification of the original Chalfejeff method (Barnard, 1932). Samples of hemin purchased from various other sources were found to contain extraneous nitrogen, so that, on alkaline solution, they would be reduced by dextrose without the necessity of the presence of nitrite.

water bath; the evaporating dish furnishes a background which facilitates reading the change in color on reduction of the reagent.

Reduction is signified by a rather sudden appearance of a scarlet color; the time interval of change from the greenish of the unreduced pigment is sufficiently short to permit a readable end point for the reaction.

Fig. 1 shows the interval from the time of immersion to that of reduction of various concentrations of dextrose. Where it is desired to conduct a quantitative determination on the basis of the addition of the test solution to the reagent at the boiling point, a graph constructed on this basis shows a shift of the entire curve to the left. This method of determination is not as convenient as that for which the graph is shown.



In urines of normal reducing power, reduction will occur in from fifteen to twenty minutes of boiling. Five minutes has been the arbitrary time limit set upon this reagent, for if reduction does not occur within this time, the urine can be regarded as being without a quantity of reducing sugar of pathologic importance.

The reagent may be boiled directly over the free flame, as is the routine procedure for use with most of the qualitative copper reagents. Eight drops of urine are added to the boiling reagent, boiling is continued for one minute, and the mixture allowed to cool. The absence of reduction after a five-minute interval constitutes a negative reaction.

REFERENCES

Barnard, R. D.: A Cyanhematin Standard for the Sahli Hemoglobinometer, J. Lab. & Clin. Med. **17:** 824, 1932.

Barnard, R. D.: Nitrite Ferrihemochromogen. Unpublished data.

Bertin-Sans, H., and de Montessier, J.: Hemochromogen, Compt. rend. Acad. de biol. **114:** 923, 1892.

TOLERANCE OF DIABETIC PERSONS FOR DEXTROSE DURING VARIOUS TIMES OF THE DAY*

MAX WISHNOFSKY, M.D., ARTHUR P. KANE, M.D., WILLIAM C. SPITZ, M.D., AND CHARLES S. BYRON, M.D., BROOKLYN, N. Y.

THE question of the tolerance of diabetic persons for dextrose during various times of the day is one of considerable scientific and practical importance. Although this problem has been studied by many students of diabetes, it can hardly be considered as conclusively settled. In this paper, we shall review and discuss the literature on the subject and present some experimental work.

The review of the literature may well be prefaced by the response to a query in which the editor of the *Journal of the American Medical Association*[1] answered in part as follows: "That the diabetic patient is at his worst in the morning, better at noon and best of all in the evening, is almost a proverb. In the morning his storage of carbohydrate is low and, as all agree, whether in diabetes or in health that is the time he utilizes carbohydrate least well; at noon and by the night meal necessary quantities of carbohydrate have been brought into action and are being metabolized and in consequence the entire machine is running more normally. Many a diabetic patient can take ten or twenty grams of carbohydrate on retiring and wake up sugar free."

The study of the blood sugar in diabetic persons during the day and night is of great importance. Pétren[2] was one of the first to observe the drop in concentration of blood sugar during the day in persons with diabetes. In 10 diabetic persons he found that the blood sugar one and one-half hours after the midday repast may even be lower than the "fasting level" at 8 A.M. He found further, that at certain hours of the day, the blood sugar may not rise at all after the ingestion of food containing starch. The most complete work on this subject is by Hatlehol,[3] who concluded as follows: During fasting the blood sugar curve does not exhibit a continuous decline, but is subject to oscillations, its fall from morning to evening being followed by a rise during the night (Fig. 1). This may be termed the paradoxical rise in blood sugar. It occurs in severe diabetics; not in the mild cases. It is not influenced by the character of the preceding diet, nor by food administered during the day. It is not due to muscular inactivity or the giving of meals during the night. It may be assumed that this phenomenon is due to sleep or some factor acting simultaneous with sleep. Vesa[4] has fully confirmed the findings of Hatlehol in the fasting diabetic person, and believes that it is not due to sleep but to an insulin-adrenalin mechanism. He contends, however, that the paradoxical rise is rendered indefinite by meals.

*From the Medical Service of Dr. Edmund L. Shlevin and the Department of Basal Metabolism of the Jewish Hospital, Brooklyn.

Received for publication, July 19, 1939.

Foster[5] found that in normal individuals a second dose of dextrose given one to one and one-half hours after the first caused little or no rise in blood sugar. Hamman and Hirschman[6] concluded from their experiments as follows: (1) The ingestion of dextrose in normal individuals in some way stimulates the mechanism of carbohydrate disposal so that the repeated ingestion of the same amount causes a less marked hyperglycemia. (2) The same stimulating effect is noted in diabetes; the second dose is followed by a less marked hyperglycemia and glycosuria. However, the difference between the effects of the two doses is less marked than in normal persons and varies in different stages of the disease. Possibly, when the diabetes is very severe, the difference may completely vanish. It should be noted that the second dose (each was usually 100 Gm.) was given from two and one-half to three hours after the first; that is, when the glycemic reaction from the first dose was declining or had just declined.

Several investigators had made similar observations previously. Most of them maintained that the fasting liver or pancreas is not ready for glycogen formation. They postulated that the hyperglycemia resulting from the first dose initiates the glycogen-forming mechanism which causes a fall in the blood sugar, and that this mechanism, not having subsided when the second dose is given, tends to prevent so marked a blood sugar concentration after that dose.

Jonas, Miller, and Teller[7] studied all-day blood sugar curves in nondiabetic and diabetic individuals receiving meals equally divided. They noted that the highest blood sugar and urine sugar occurred after breakfast, only when the interval between meals was short. They postulated that this may not be so when the interval is long. Sakaguchi and Sato[8] found that when identical meals were given three hours apart to normal individuals and mild diabetic persons, the second blood sugar curve was lower than the first. When the interval was six to eight hours, however, the blood sugar curves were the same. In more severe cases of diabetes, even when the interval was only three hours, the second blood sugar curve was not lower than the first. MacLean and de Wesselow[9] observed that when two doses of dextrose were given four hours apart to normal individuals, the blood sugar reaction to the second dose was independent of the first.

A decade ago Forsgren originated the concept that the liver works rhythmically in the performance of its various functions. Since then he has written extensively on this subject. He contends that the rhythmic function of the liver is not an isolated phenomenon, but an expression of rhythmicity of the entire vegetative system. This conception, as far as carbohydrate metabolism is concerned, Forsgren based on experiments he performed on rabbits.[10] He found that in these animals, glycogen accumulated during the night disappeared again the following morning. This phenomenon was entirely independent of food intake, and has been fully confirmed by Agren, Wilander, and Jorpes[11] who, as a result of extensive experiments performed on rats and mice, concluded as follows: There are cyclic changes in the glycogen content of the livers of rabbits, rats, and mice which are, to a large extent, independent of the intake

of food. Glycogen accumulates in the liver during the night and disappears to some extent during the next morning. Similar periodical changes occur, though to a minor extent, in muscles also.

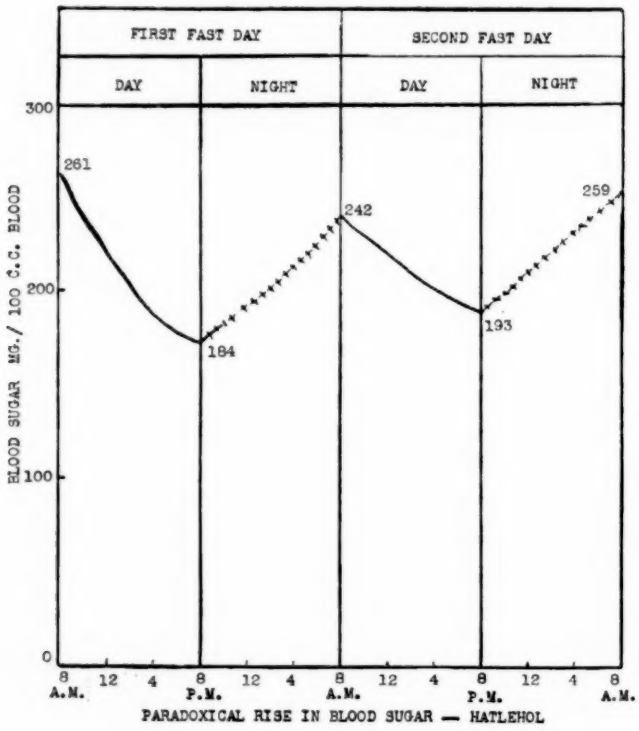


Fig. 1.

Chiefly with these findings as a basis, Forsgren has evolved the conception of the rhythmic function of the liver in the human being as far as glycogen metabolism is concerned. He contends that during the night and early morning hours the secretory or glycogenolytic phase occurs, which is associated with a rise in blood sugar and a decreased sensitivity to insulin. At noon the glycogenetic phase sets in; this is associated with a fall in blood sugar and an increased sensitivity to insulin, independent of meals. However, meals given during the glycogenolytic phase produce a greater rise in blood sugar than those given during the glycogenetic phase. In most individuals the glycogenetic phase occurs between 11 A.M. and 5 P.M., but may vary.

Möllerstrom[12] and Hopmann and Martini[13] are strong exponents of Forsgren's conception of the rhythmic function of the liver. Experimentally, the latter gave two 30 Gm. doses of dextrose to each of 6 diabetic persons. Both doses were given on the same day; one at 3 A.M., the other at 12 A.M. The blood sugar was studied from 3 A.M. to 7 A.M., and from 12 A.M. to 4 P.M. In 3 cases the blood sugar curves in the afternoon were identical with those in the morning; in the other 3 cases the blood sugar curves in the morning were higher.

An analysis of the literature leads one to conclude that the tolerance of persons with diabetes for dextrose during various times of the day can hardly be considered definitely settled. The following criticism may be offered of the experiments in which repeated doses of carbohydrate were given. In the first place, in the majority of cases the interval between doses was short, so that the second dose of dextrose was given when the glycemic reaction from the first was declining or had just declined. Furthermore, only a few cases were studied. Sakaguchi and Sato studied the reaction when the interval between doses was sufficiently long, but this was in very few instances. In the second place, the subjects all had mild diabetes. Conclusions drawn from results obtained from such cases are not applicable to persons with severe diabetes.

Forsgren's conception of the endogenous rhythmicity of the liver is based almost entirely on animal experiments in which the glycogen content of the liver was studied during various times of the day. It must be said that such evidence is insufficient for the establishment of a conception as to the function of the liver in the human being. Hopmann and Martini's experiments, in support of Forsgren's idea, are insufficient in number (only 6 cases were studied) and show inconclusive results.

METHOD AND RATIONALE OF PROCEDURE

Experiments were performed on 20 diabetic persons with varying degrees of severity. The subjects were all adult, ambulatory females. Only those taking unmodified insulin or no insulin were studied. Patients on protamine zinc insulin were excluded because of the prolonged action of the drug and the undesirability of discontinuing insulin for long periods of time. The effect of exogenous insulin was entirely eliminated by having those taking the unmodified insulin discontinue its use for from thirty-six to forty-eight hours before the beginning and during the entire period of experimentation.

To each of 20 persons with diabetes 70 Gm. of dextrose in a 15 per cent aqueous solution were given at 8 A.M. after a fast of fourteen hours. Specimens of blood (venous) were taken at the fasting level, immediately before ingestion, and one, two, and three hours after the ingestion of the dextrose. The sugar content of the blood was determined by the Folin-Wu method. Urine was collected one and one-half and three hours after the ingestion of dextrose, and its sugar content determined by the Benedict method.

Two days later, at 7 A.M., each patient received a meal consisting of two slices of bread, two eggs, and one orange. (The food value of this meal is carbohydrate 46 Gm., protein 18 Gm., and fat 12 Gm.) They did not receive anything else except water until 2 P.M., at which time they were given 70 Gm. of dextrose in a 15 per cent aqueous solution. The specimens of blood and urine were collected at the same periods as when the dextrose was given in the morning two days previously. In the interval between the tests the patients received their customary diets.

It will be noted that the second dose of dextrose was given seven hours after breakfast. It is reasonable to assume that the breakfast had been completely digested and absorbed, and that the glycemic and glycosuric reactions produced by it had long since subsided.

The question of the validity of comparing blood sugar curves of tests performed two days apart has been answered fully in several previous papers in which we used similar methods of experimentation. Briefly, it may be stated that dextrose tolerance tests, utilizing equivalent loads of carbohydrate performed on a series of diabetic persons two days apart at the same time in the morning, reveal identical glycemia and glycosuria.[14] Any differences in the blood sugar curves in the afternoon, as compared to those in the morning two days previously have, therefore, unqualified significance.

ANALYSIS OF RESULTS

Inspection of the concentration of blood sugar both before and after the ingestion of dextrose in the afternoon, as compared with that in the morning, revealed that persons with mild diabetes reacted in a manner strikingly different from those with the severe form. Accordingly, it became necessary to divide the cases into two groups. Purely arbitrarily, those with a fasting blood sugar below 200 mg. per 100 c.c. of blood were classed as mild or moderately severe cases, and those with a value above 200 mg. per 100 c.c. of blood as severe. The concentrations of blood sugar and amounts of dextrose in the urine before and after the ingestion of dextrose in the morning and in the afternoon, in persons with mild and moderately severe diabetes are recorded in Table I. In Table II similar data are recorded for the severe cases. The data in the tables will be analyzed under the following headings: 1. Concentration of blood sugar at the preingestion level. 2. Concentration of blood sugar after the ingestion of dextrose. 3. Urine sugar.

1. *Concentration of Blood Sugar at the Preingestion Level.*—In Table I (mild and moderately severe diabetes) it will be observed that the values in 8 out of 10 cases are somewhat higher at 8 A.M. than at 2 P.M. The mean difference is only + 7. The severe cases react in a strikingly different manner. In all the cases the values are decidedly lower at 2 P.M. and the mean difference is + 63.

2. *Concentration of Blood Sugar After the Ingestion of Dextrose.*—In the mild and moderately severe cases (Table I) the values two hours postcibal are higher in the afternoon in 8 out of 10 cases. The same holds true for 7 out of 10 cases at three hours postcibal. This is in contrast to the values at the preingestion level where the reverse is true; namely, in 8 out of 10 cases, the value is higher at 8 A.M.

In the severe cases (Table II) a distinctly different picture presents itself. In practically every case, at all levels, namely one, two, and three hours postcibal, as well as at the preingestion level, the concentrations of blood sugar in the afternoon are lower than the corresponding values in the morning. Thus the blood sugar curve in the afternoon, while it parallels that in the morning, is at a distinctly lower level.

3. *Urine Sugar.*—In persons with mild and moderately severe diabetes the urine sugar values were studied in only 7 cases (Table I). It will be observed that in 6 out of 7 cases the values are higher in the morning in the urines collected zero to ninety minutes postcibal. This may partly be explained by

TABLE I

CONCENTRATION OF DEXTROSE IN THE BLOOD AND QUANTITY OF DEXTROSE IN THE URINE OF PERSONS WITH MILD AND MODERATELY SEVERE DIABETES IN THE MORNING AND IN THE AFTERNOON BEFORE AND AFTER THE INGESTION OF 70 GM. DEXTROSE

CASE NO.	BLOOD SUGAR (PER 100 C.C. BLOOD)								URINE SUGAR (GM.)			
	FASTING LEVEL		1 HOUR POSTCIBAL		2 HOURS POSTCIBAL		3 HOURS POSTCIBAL		0 TO 90 MIN. POSTCIBAL		90 MIN. TO 3 HOURS POSTCIBAL	
	8 A.M.	2 P.M.	9 A.M.	3 P.M.	10 A.M.	4 P.M.	11 A.M.	5 P.M.	A.M.	P.M.	A.M.	P.M.
1	139	180	260	328	292	339	247	284				
2	153	127	248	231	258	263	230	211				
3	105	96	184	217	164	278	100	213				
4	156	143	306	274	328	345	274	284	2.1	0.0	1.5	0.0
5	182	124	304	292	318	305	204	183	1.4	0.7	1.6	1.7
6	165	139	308	367	312	308	247	217	1.6	1.0	2.4	0.7
7	196	188	350	312	356	381	246	274	5.1	1.9	6.4	3.6
8	148	132	282	218	305	325	237	274	1.7	0.8	5.1	4.2
9	127	109	274	254	252	282	179	228	1.5	0.7	1.1	1.3
10	96	160	286	339	325	392	263	305	1.2	1.6	3.4	4.3
Means	147	140	280	280	291	322	223	247	2.1	0.96	3.1	2.1
Difference between means*	+7		±0		−31		−24		+1.04		+1.0	

*The sign is taken as plus when the value in the morning is greater and as minus when the value in the afternoon is greater.

TABLE II

CONCENTRATION OF DEXTROSE IN THE BLOOD AND QUANTITY OF DEXTROSE IN THE URINE OF PERSONS WITH SEVERE DIABETES IN THE MORNING AND IN THE AFTERNOON BEFORE AND AFTER THE INGESTION OF 70 GM. DEXTROSE

CASE NO.	BLOOD SUGAR (MG. PER 100 C.C. BLOOD)								URINE SUGAR (GM.)			
	FASTING LEVEL		1 HOUR POSTCIBAL		2 HOURS POSTCIBAL		3 HOURS POSTCIBAL		0 TO 90 MIN. POSTCIBAL		90 MIN. TO 3 HOURS POSTCIBAL	
	8 A.M.	2 P.M.	9 A.M.	3 P.M.	10 A.M.	4 P.M.	11 A.M.	5 P.M.	A.M.	P.M.	A.M.	P.M.
1	312	245	455	364	513	408	409	342	12.3	6.4	25.2	16.9
2	216	196	390	342	460	377	351	288	1.7	1.4	14.9	3.1
3	350	298	392	360	420	410	384	330	8.4	7.1	12.3	9.6
4	242	151	378	294	400	328	262	206	7.0	4.4	9.6	4.9
5	208	133	400	351	312	276	216	183	4.2	2.0	2.0	1.5
6	203	179	392	381	385	388	298	306	5.8	6.2	8.7	12.2
7	235	138	449	305	540	367	476	303	0.5	0.5	3.0	1.8
8	237	160	385	348	449	408	342	312	5.4	1.6	2.3	6.2
9	267	216	364	348	412	392	315	310	4.7	4.5	8.8	6.4
10	261	180	430	435	435	440	345	339	8.8	5.3	7.0	4.9
Means	253	190	404	353	433	379	340	292	5.88	3.94	9.38	6.75
Difference between means*	+63		+51		+54		+48		+1.92		+2.63	

*The sign is taken as plus when the value in the morning is greater and as minus when the value in the afternoon is greater.

the fact that the blood sugar is higher at 8 A. M. than at 2 P.M. in these cases. In 4 out of 7 cases the values are higher in the morning in the urines collected ninety minutes to three hours postcibal. In the persons with severe diabetes the values are higher in the morning in 8 out of 10 cases.

COMMENT

The primary purpose of this work was to determine the tolerance of diabetic persons for dextrose during various times of the day. However, the method of procedure and the results obtained make possible the elucidation of various other related problems. The comment will, therefore, concern itself with a discussion of the relationship of the results to the following:

1. Tolerance of diabetic persons for dextrose during various times of the day.
2. Effect of repeated doses of dextrose on the blood sugar curve.
3. Forsgren's conception of the rhythmic function of the liver.
4. Hatlehol's so-called "paradoxical rise in blood sugar."

1. *Tolerance of Diabetic Persons for Dextrose During Various Times of the Day.*—Our work is limited to a study of the tolerance of diabetic persons for dextrose in the morning as compared with the afternoon. The prevailing conceptions are fully discussed in the review of the literature. The results of our experiments on 20 persons with diabetes of varying degrees of severity are sufficiently clear-cut to permit the drawing of categorical conclusions. The findings indicate that no general law applies, but that a definite difference exists between persons with mild and severe diabetes. In mild and moderately severe diabetes the tolerance for dextrose in the morning is equal to, if not greater than, that in the afternoon. In severe diabetes an entirely different picture presents itself. Here the tolerance in the afternoon is significantly greater than in the morning. The reason for this difference is in the present state of our knowledge inexplicable, and it would be futile to theorize as to the mechanism involved.

The prevailing impression is that the difference between mild and severe diabetes is purely a quantitative one. The difference in tolerance for dextrose in the afternoon as compared to the morning between the two groups is so marked, however, that the suggestion may be made that a qualitative difference is also present.

2. *Effect of Repeated Doses of Dextrose on the Blood Sugar Curve.*—Foster,[5] Hamman and Hirschman,[6] and Sakaguchi and Sato[8] found that in normal individuals and in persons with mild diabetes a second dose of dextrose produced a lower glycemic reaction than the first one. The explanation given is that the fasting liver or pancreas is not ready for glycogen formation. It was noted above that in their experiments the second dose was given when the glycemic reaction from the first dose was declining or had just declined. Maclean and de Wesselow,[9] in normal individuals, and Sakaguchi and Sato,[8] in diabetic persons, observed that when the interval was long, the glycemic reaction to the second dose was independent of the first one. In our series of persons with mild diabetes, the interval between breakfast and the ingestion of dextrose was seven hours. The glycemic reaction resulting from the breakfast had long since subsided. The blood sugar curve in the afternoon is at least equal to, if not higher than, the one obtained in the morning after the ingestion of an equal amount of dextrose. These findings are in complete accord with those of Maclean and de Wesselow, and Sakaguchi and Sato. The conculsion

to be drawn is that the capacity of the fasting liver (fourteen hours' fast) to store glycogen in the morning is no worse than in the afternoon, seven hours after the individual has had a breakfast containing a moderate amount of carbohydrate.

3. *Forsgren's Conception of the Rhythmic Function of the Liver.*—Briefly, Forsgren conceives as follows: 1. During the night and early morning, the secretory or glycogenolytic phase, which is associated with a rise in blood sugar and a decreased sensitivity to insulin, occurs. 2. At noon the glycogenetic phase sets in, and this is associated with a fall in blood sugar and an increased sensitivity to insulin. 3. Meals given during the glycogenolytic phase produce a greater rise in blood sugar than those given during the glycogenetic phase.

If Forsgren's conception is tenable, it should apply to all diabetic persons, and especially to persons with mild diabetes, since it is in this group that the capacity to store glycogen is greatest. Our findings indicate, however, that no general law applies, and that it is in the mild cases that the tolerance for dextrose in the morning is at least equal to, if not greater than, in the afternoon. Accordingly, Forsgren's conception must be considered untenable in diabetic human beings.

4. *Hatlehol's So-Called Paradoxical Rise in Blood Sugar.*—As previously noted, Hatlehol found that the blood sugar falls decidedly during the day in severe diabetic persons, but only slightly in mild cases. He also noticed that this fall is uninfluenced by meals. A study of the preingestion blood sugar values (Tables I and II) at 8 A.M. and 2 P.M. fully confirms Hatlehol's findings.

CONCLUSIONS

1. The tolerance of diabetic persons for dextrose in the afternoon, as compared to the morning, was studied. It was found that no general law applies. In mild and moderately severe forms of diabetes the tolerance in the morning is equal to, if not greater than, that in the afternoon. In persons with severe diabetes, however, the tolerance is distinctly better in the afternoon.

2. The capacity of the liver to store glycogen in the morning, after a fast of fourteen hours, is no worse than in the afternoon, seven hours after a breakfast containing starch.

3. The findings made here make Forsgren's conception of the rhythmic function of the liver untenable as far as carbohydrate metabolism is concerned.

4. Hatlehol's findings of the paradoxical rise in blood sugar is in part confirmed.

REFERENCES

1. Insulin in Diabetes, Queries and Minor Notes, J. A. M. A. **110:** 1129, 1938.
2. Flaum, A., and Pétren, K.: Observations sur la courbe du sucre sanguin chez les diabètiques au cours de la journée, Compt. rend. Soc. de biol. **93:** 380, 1925.
3. Hatlehol, R.: Paradoxical Rise of the Blood Sugar Concentration in Diabetes Mellitus, Acta med. Scandinav., Suppl. 8, 1924.
4. Vesa, A.: Unter Anwendung von Zweistündlichen bei Tag und Nacht Entnommenen Blutzucker und Harnproben, Acta med. Scandinav. Supp. **57:** 134, 1934.
5. Foster, G. L.: Interpretation of Blood Sugar Phenomena Following Ingestion of Glucose, J. Biol. Chem. **55:** 303, 1923.
6. Hamman, L., and Hirschman, I. I.: Effects Upon the Blood Sugar of Repeated Ingestion of Glucose, Bull. Johns Hopkins Hosp. **30:** 308, 1919.
7. Jonas, L., Miller, T. G., and Teller, I.: All Day Blood Sugar Curves in Nondiabetic Individuals and in Diabetic Patients With and Without Insulin, Arch. Int. Med. **35:** 289, 1925.

8. Sakaguchi, K., and Sato, K.: Über den Einfluss der Zeit der Nahrungsaufnahme auf die alimentäre Hyperglykämie, Mitt. d. a. med. Fakult. d. k. Univ. zu Tokyo **23:** 373, 1920.
9. Maclean, H., and de Wesselow, O. L. V.: Estimation of Sugar Tolerance, Quart. J. Med. **14:** 103, 1921.
10. Forsgren, E.: (a) Über die rhythmische Funktion der Leber und ihre Bedeutung für den Kohlehydratstoffwechsel bei Diabetes und die Insulinbehandlung, Klin. Wchnschr. **1:** 1110, 1929. (b) Über Leberfunktion, Harnausscheidung und Wasserbelastungsproben, Acta. med. Scandinav. **76:** 286, 1931. (c) Über Leberfunktion und Insulinempfindlichkeit, Klin. Wchnschr. **2:** 1429, 1932.
11. Agren, G., Wilander, O., and Jorpes, E.: Glycogen Content of the Liver and Muscles of Rats and Mice, Biochem. J. **25:** 777, 1931.
12. Möllerström, J.: Über die 24-Stunden-Periodizität des inneren Stoffwechsels, und die rhythmische Funktion der Leber, sowie deren Bedeutung für die Insulinbehandlung bei Diabetes, Deutsches Arch. f. klin. Med. **173:** 484, 1932.
13. Hopmann, R., and Martini, H.: Der Tag-Nachtrhythmus des Diabetes mellitus, Ztschr. f. klin. Med. **129:** 70, 1935.
14. Wishnofsky, M., and Kane, A. P.: The Effect of Equivalent Amounts of Dextrose and Starch on Glycemia and Glycosuria in Diabetes Mellitus, Am. J. M. Sc. **189:** 545, 1935.

DIFFICULTIES ENCOUNTERED IN A TEST FOR STANDARDIZATION OF TOXIN USED AGAINST SCARLET FEVER*

BETTY S. KOLCHIN, NEW YORK, N. Y.

THE Dick reaction as a diagnostic method and as a guide to immunity has been accepted as a routine procedure by the attending staffs of hospitals for contagious diseases. However, certain anomalies of the test are known to exist. The discrepancies become particularly disturbing when they appear during the standardization of preparations used for testing and immunizing purposes. The source of the irregularities is not limited to one single factor. We are here presenting a limited group of observations in the hope that it may help to remove some of the complicating factors in standardization tests of preparations used for prevention of scarlet fever.

HISTORICAL

In 1925 Joe,[1] in a comprehensive clinical study of the Dick test, observed the changing of a positive reaction to a negative, or vice versa, when readings were made eight hours and again twenty-four hours after injections. In the same year, Zoeller and Manoussakis reported a study of the influence on local immunity of repeated injections with one skin test dose. He pointed out that in some individuals a refractory condition of the skin developed, where the negative reaction was not associated with the development of general immunity. O'Brien[3] in 1930, reporting a careful study by Okell and Parish of "The Titration of Scarlet Fever Toxin," concluded that the difficult matching of two toxins would be possible only by a careful choice of subjects and by several readings of the reactions at different intervals after the injection. McGibbon[4] in 1934, from his observations on the Dick test in patients and convalescents, concluded that readings within the first sixteen hours would

*From the Bureau of Laboratories, Department of Health, New York.
Received for publication, July 28, 1939.

give a higher percentage of positives than readings made twenty-four hours after injections. Smiley[5] in 1935 suggested that inconsistencies were due to previous injections altering the response of the skin to subsequent skin tests. Ker[6] in 1937, in "Some Observations on the Dick Test" made in a study prompted by a run of anomalous reactions, stated that "the best interval to allow between performing and reading is probably between 12 and 18 hours." He also stressed the importance of the proper site for injecting. More recently, the Dicks[7] showed that 10 skin test doses injected intradermally produce in some individuals a local skin immunity regardless of their general susceptibility to scarlet fever.

OBSERVATIONS

For some time we have been experiencing difficulties while standardizing our testing and immunizing preparations on skins of institutional children. Much irregularity has been noticed, both in individual tests performed on a group of children on the same date and successive tests with the same preparations on different dates. In the first case, one preparation would appear much stronger than the standard on some children and weaker than the standard on others in a disproportionate manner. In the second case, a preparation would appear stronger than the standard on one date and weaker on another. The cause of these distressing facts was not understood, but was eventually suspected. As all the technical factors in these tests were carefully checked, we directed our attention to the medium on which the tests were performed, that is, the children. The possibility of a peculiarly modified local skin condition was considered. With this in mind, we planned the tests.

EXPERIMENTAL

I. Discrepancies in Simultaneous Reactions in Different Skin Areas of the Same Individual.

Experiment 1.—Two Dick preparations made up simultaneously in exactly the same manner in large gallon bottles were tested at the same time against the standard toxin on two groups of children, 15 in each group. Each toxin preparation to be tested and the standard Dick preparation were injected twice in each child. The results were surprisingly enlightening. One preparation was considerably stronger than the standard on one arm and weaker on the other; or the same preparation would give a stronger reaction on one arm than on the other, or would be completely negative on one and strongly positive on the other. The standard preparation itself gave different reactions in two different areas injected at the same time on the same individual. The range of variation was from strongly positive to weakly positive or from strongly positive to completely negative in about 50 per cent of all positive reactors. Protocol 1 of the first test illustrates this fact. A similar test was repeated with three other preparations following the same plan and the results were very much the same.

A circumstance should be pointed out that approximately coincided in time with the foregoing observation: the transfer of our testings to an institution where children spend many years and are subjected to repeated injections for diagnostic, immunizing, or investigative purposes (diphtheria, tuberculosis,

PROTOCOL 1

THREE PREPARATIONS OF DICK TOXIN EACH INJECTED SIMULTANEOUSLY ON BOTH ARMS

GROUP I	DICK PREP. NO. 88		DICK PREP. NO. 89		STANDARD DICK PREP. 1 S.T.D.*	
H. L.	16 × 20	12 × 15	11 × 10	–	–	13 × 13
C. T.	–	–	–	–	–	–
D. L.	–	–	–	–	–	–
J. M.	25 × 42	22 × 25	11 × 15	13 × 17	11 × 15	15 × 20
G. F.	–	10 × 12	–	–	–	9 × 9
S. S.	–	–	–	–	–	–
A. L.	10 × 16	15 × 16	15 × 15	15 × 18	–	20 × 20
A. M.	–	–	–	–	–	–
C. S.	16 × 19	20 × 19	16 × 17	20 × 30	9 × 10	20 × 16
F. Z.	15 × 20	–	12 × 14	12 × 15	–	11 × 14
A. S.	20 × 24	21 × 26	20 × 24	17 × 21	10 × 10	21 × 25
I. C.	–	–	–	–	–	–
R. N.	–	12 × 17	–	10 × 8	–	10 × 12
E. N.	–	–	–	–	–	–
M. Z.	–	–	–	–	–	–

GROUP II	DICK PREP. NO. 88		DICK PREP. NO. 89		STANDARD DICK PREP. 1 S.T.D.*	
J. G.†						
F. B.	–	–	–	–	–	–
F. D.	10 × 12	–	12 × 12	12 × 11	12 × 11	–
B. F.	–	–	–	–	–	–
R. K.	15 × 20	9 × 10	14 × 14	15 × 15	10 × 9	20 × 20
M. S.	–	–	–	–	–	–
A. W.	–	–	–	–	–	–
W. G.	–	–	–	–	–	–
J. G.	14 × 20	12 × 12	–	11 × 14	10 × 10	–
R. H.	–	–	–	–	–	–
B. W.	–	–	–	–	–	–
J. G.	26 × 22	15 × 16	12 × 11	18 × 20	11 × 14	13 × 18
J. B.	12 × 15	–	–	10 × 10	–	–
T. M.	–	–	–	–	–	–
J. P.	–	–	–	–	–	–

Each double column represents the same preparation in corresponding areas of left and right arm.

*Skin test dose.

†Reactions were not read.

– = No reaction.

etc.). Whether these result in a temporary state of local immunity to the streptococcal toxin or, on the contrary, in increased local sensitivity (specific or nonspecific), we leave for subsequent discussion. Here we shall only mention that either of these induced conditions might be the cause of the discrepancies under discussion.

II. Discrepancies in Results in Two Successive Readings at Eight Hours and Twenty-Two Hours After Injection.

The possibility of the existence of another factor creating the described discrepancies, a factor that may add to the distorting influence of repeated injections, occurred to us for the following reason: Some normal children, such as those in private practice, where no repeated injections are likely to have taken place, sometimes give irregular and contradictory skin test readings. In view of the peculiarly early appearance of the positive reactions to the hemolytic streptococcus toxin, it seems possible that in some individuals an early appearance of a positive reaction is followed by its early disappearance and is thus missed at the established time of reading—within 24 hours. With this possibility in view, another experiment was carried out (Protocol 2).

Experiment 2.—Forty-three individuals, most of them known to be positive reactors, were inoculated with a total of 258 injections (3 injections on each arm of every individual) at 10 to 11 A.M. The results were observed and measured twice: within 7 to 8 hours and again the next morning—within 21 to 22 hours after the injections were made. The comparison of the two series of readings confirmed the supposition that time was a factor in the irregularities observed. The results were as follows: 43 (Table I, first test) individuals received 6 injections each; of these, 23 individuals gave negative reactions to all 6 injections in 7 to 8 hours, and remained negative during the second reading in 21 to 23 hours; 10 individuals gave all positive reactions to the 6 injections and these too remained unchanged in 21 to 23 hours. This gives a total of 33 individuals (out of 43) with unchanged reactions for two readings—whether positive or negative. However, the remaining 10 individuals (out of 43) gave reactions that reversed between the two readings, these reversals working both ways. Two individuals changed reaction from negative in 7 to 8 hours to positive in 21 to 22 hours, and 8 individuals reversed reactions in the opposite direction, being positive at the first reading in 7 to 8 hours and becoming completely negative at the time of the second reading in 21 to 22 hours. (The change of reactions in an individual affects one injection or more than one, and occasionally all 6.)

TABLE I

SUMMARY OF 4 TESTS

NUMBER OF TEST	NUMBER OF INDIVIDUALS IN EACH TEST	NUMBER OF INDIVIDUALS WITH ALL REACTIONS UNCHANGED IN BOTH READINGS				NUMBER OF INDIVIDUALS WITH CHANGED REACTIONS IN AT LEAST ONE INJECTION			
		ALL NEGATIVE	ALL POSITIVE	TOTAL UNCHANGED	PER CENT	− TO +	+ TO −	TOTAL CHANGED	PER CENT
1st	43	23	10	33		2	8	10	
2nd	33	26	3	29		2	2	4	
3rd	37	26	0	26		2	9	11	
4th	47	33	6	39		8	0	8	
Total	160	108	19	127	79.5	14 (8.75%)	19 (11.9%)	33	20.6

Three additional similar tests were performed with double readings, each test representing a different type of result: Test 2 with an even proportion of "positive-to-negative" and "negative-to-positive" changes; Test 3 with a predominance of the "positive-to-negative" change (as in the first test analyzed in detail); and finally, Test 4, in which, on the contrary, an increased number of positive reactions were observed during the second reading—with none in this group of 47 individuals showing an early positive reaction changing to a negative the next morning.

Thus the four experiments represent apparently all types of constancy and changeability between readings taken at two different time intervals. The summary in Table I brings out the following: In four tests with a total of 160 individuals, 127 persons, or 79.5 per cent, gave "stable" reactions, either positive or negative, during both readings. The remaining 33 individuals, or 20.6 per cent, with changing reactions gave the following distribu-

PROTOCOL 2

Three Preparations of Dick Toxin Each Injected Simultaneously on Both Arms, With Reactions Observed in 8 and 24 Hours After Injections

	DICK PREP. NO. 195		DICK PREP. NO. 198		STANDARD DICK PREP. 1 S.T.D.*	
W. S.	18 × 15++ 12 × 14±	18 × 25 13 × 20	20 × 15++ 8 × 12±	18 × 25++ 15 × 20+	18 × 15++ 15 × 16+	18 × 25++ 17 × 20+
A. H.	13 × 16 Scratched	8 × 10± –	8 × 8 –	8 × 8 –	– –	– –
I. B.	10 × 8± 10 × 15	14 × 15± –	10 × 10 –	8 × 10 –	– –	– –
N. G.	10 × 20 15 × 24	14 × 20 14 × 23	20 × 16+ 16 × 21±	8 × 10 10 × 15±	12 × 12 10 × 12	14 × 20 17 × 20++
A. P.	– –	9 × 10 –	– –	8 × 8 –	– –	– –
S. S.	– –	– –	– –	9 × 12 –	– –	– –
H. R.	18 × 25++ 20 × 28	15 × 28 15 × 15±	18 × 25++ 20 × 17++	15 × 30++ 16 × 20±	20 × 23++ 15 × 20±	18 × 28++ 16 × 24++
E. M.	9 × 9 –	– –	– –	8 × 8 –	– –	– –
R. R.	– –	10 × 15 –	– 7 × 7	– –	– –	– –
R. C.	– –	– –	– –	– –	– –	– –
G. R.	23 × 30 –	18 × 20 15 × 18	25 × 25 18 × 21	20 × 25 20 × 20	25 × 25 12 × 12	25 × 28 20 × 25
R. H.	– 19 × 30	15 × 25 20 × 28	15 × 16 18 × 28	12 × 15 16 × 24	14 × 12 18 × 21	10 × 15 19 × 26
R. V.	– –	8 × 8 –	– –	8 × 8 –	– –	– –
J. G.	– –	7 × 10 8 × 8	– –	8 × 8 –	– –	– –
A. S.	20 × 30++ 20 × 25+	20 × 28++ 20 × 26±	20 × 30++ 20 × 25+	20 × 28++ 18 × 24±	25 × 25 18 × 20	22 × 30++ 20 × 20±
W. S.	10 × 10 –	6 × 9 –	– –	6 × 8 –	– –	– –
N. S.	15 × 15 18 × 18	22 × 30 –	14 × 12 –	20 × 26 15 × 15±	25 × 28 –	23 × 23 10 × 12±
W. M.	– –	– –	– –	– –	– –	– –
C. M.	20 × 30 20 × 30	18 × 25 19 × 25	8 × 8 –	15 × 25 19 × 24	9 × 11 9 × 11	15 × 17 15 × 16
E. V.	17 × 25 15 × 25	15 × 35 17 × 28	12 × 15++ –	20 × 30 15 × 25	20 × 30 22 × 28	20 × 25 19 × 25
R. S.	18 × 25 18 × 20	24 × 25 19 × 20	15 × 25 18 × 25	14 × 20 15 × 18±	15 × 15++ ±	20 × 20 18 × 18
T. L.	–	–	–	–	–	–
J. M.	–	–	–	–	–	–
W. B.	8 × 8	–	–	–	–	–
G. R.	12 × 12 –	– –	8 × 8 –	– –	– –	– –
W. H.	– –	– –	– –	– –	– –	– –
R. D.	25 × 32 25 × 25	20 × 30++ 20 × 30+	22 × 28 20 × 27	20 × 28++ 25 × 30±	18 × 20 18 × 18	22 × 25++ 25 × 28+

PROTOCOL 2—CONT'D

	DICK PREP. NO. 195		DICK PREP. NO. 198		STANDARD DICK PREP. 1 S.T.D.*	
R. T.	18 × 28 15 × 22	18 × 30++ 18 × 25±	16 × 26 15 × 20	18 × 25++ 16 × 20±	15 × 18+ 14 × 15±	15 × 20+ 15 × 19±
L. S.	20 × 22++ 17 × 19	18 × 25++ 18 × 25±	20 × 25++ 12 × 18±	20 × 25++ 18 × 18±	16 × 20++ 15 × 16±	20 × 25++ 18 × 23±
W. S.	23 × 22 26 × 30	15 × 15+ 15 × 15±	15 × 20++ 10 × 12±	15 × 15+ –	10 × 10 10 × 14±	15 × 15 11 × 14±
S. M.	7 × 7 –	– –	6 × 6 –	± –	9 × 10 –	± –
V. M.	20 × 23++ 20 × 26+	20 × 27++ –	20 × 26++ 20 × 28+	25 × 30++ 25 × 28±	20 × 22++ 20 × 22+	20 × 25++ 25 × 30+
S. G.	– –	– –	– –	– –	– –	– –
S. W.	– –	– –	– –	– –	– –	– –
E. B.	17 × 22 25 × 24++	10 × 20 16 × 20	20 × 24 18 × 20	18 × 29 20 × 24	15 × 18 17 × 20	23 × 20 25 × 25
S. S.	8 × 8 15 × 15	– –	– 10 × 14	– 11 × 13	9 × 10 13 × 19	– 12 × 12
M. M.	– –	8 × 8 –	8 × 10 –	– –	6 × 8 –	– –
B. S.	22 × 27 22 × 28	16 × 25++ 16 × 22±	16 × 29++ 17 × 25+	20 × 35++ 20 × 30+	18 × 23 18 × 22	18 × 19 20 × 22
R. C.	– –	– –	– –	– –	– –	– –
V. H.	– –	– –	7 × 7 –	– –	7 × 7 –	– –
B. Br.	– –	7 × 7 –	– –	8 × 8 –	8 × 8 –	– –
A. J.	15 × 15 –	18 × 18 –	15 × 15 –	19 × 20 –	17 × 15 –	17 × 17 –
A. L.	– –	– –	– –	– –	– –	– –

Each double column represents simultaneous injections with the same material in corresponding areas of left and right arm.

Upper and lower results on each line represent the 8 hours', and 21 to 22 hours' readings, respectively.

*Skin test dose.

tion: 14, or 8.75 per cent, were negative at the end of the first day, turning to positive the next morning; 19, or 11.9 per cent, showed the opposite positive during the early reading (7 to 8 hours) and disappearing to totally negative at the last reading in 21 to 22 hours. The 11.9 per cent includes cases with reactions that changed from positive to negative; in some of them only one reaction changed; in others, 6 reactions changed, while in most of them, the number of positive-to-negative reversals was between these two extremes. Considering that in an ordinary Dick test any one of these six sites might have been selected for the injection, even the cases in which there is only one change of reaction should be considered significant.

DISCUSSION

The observed anomalies in a toxin-standardization test bear an analogy to certain peculiar phenomena occurring on the skin during an acute disease—whether caused by *Streptococcus hemolyticus* or by an unrelated infection.

In 1925 Zingher[8] described observations in scarlet fever patients in whom the site of a previous Dick positive reaction appeared entirely blanched out

amid the surrounding intensely red scarlatinal rash. He ascribed this to local cellular immunity caused by a previous injection of the Dick testing dose. He also gave the same interpretation to a similar observation on scarlet fever patients by Moriwaki.[9] On the other hand, evidence of an opposite nature has been seen in patients with either scarlet fever or acute diseases of a different origin than *Streptococcus hemolyticus*, such as staphylococcus or measles. It was brought out by Ferry,[10] Toomey,[11] and Moriwaki[9] that the site of a previous Dick reaction becomes greatly intensified in color during a subsequent attack of scarlet fever, measles, or acute staphylococcus infection. These authors agreed with Toomey[11] that the observed phenomenon was caused by a nonspecific sensitization of the skin in a previous Dick test. Confirming these two opposite types of observations, Goodall[12] in 1936 presented an interesting case, which combined the two features described: During an attack of scarlet fever one previous site of a Dick reaction was blanched out, while another (of an earlier date) on the same patient reappeared as a positive reaction sharply separated from the scarlet fever rash by its intensified crimson red color.

The described phenomena refer to observations of a site of an old Dick test during a subsequent illness caused either by *Streptococcus hemolyticus* or by different infection (staphylococcus, measles). However, these observations seem to throw light on the paradoxical results in a Dick test itself. If a previous injection with one skin test dose is sufficient to prevent the appearance of rash in the presence of the circulating toxin in a scarlet fever patient, how much more chance there must be for the prevention of a Dick positive reaction in a nonimmune healthy person when only one skin test dose of testing toxin is subsequently injected. On the other hand, an intensification of a previous Dick reaction in the presence of measles or a staphylococcus infection suggests nonspecific sensitization analogous to a positive reaction in a generally immune healthy person, caused by previous injections with unrelated antigens. The evidence in our own experience points to the same cause. Repeated injections for diagnostic and immunizing purposes with different antigens used in a large institution for diagnostic and immunizing purposes modify the normal sensitivity of the skin in two possible ways: some areas (in certain children) become refractive to subsequent injections with the Dick material, causing false negative reactions; others become unduly responsive, causing false positive reactions.

With regard to variations of results produced by an early fading of a positive reaction, the reason is not very clear. There is a possibility that the same factors causing pseudonegative or pseudopositive reactions will result in an early fading of a positive reaction—if either the local cellular immunization of the skin by a previous injection was incomplete or its local sensitization by a nonspecific substance was incomplete. In either case a reaction of brief duration is conceivable.

SUMMARY AND CONCLUSIONS

1. Irregularities in skin reactions have caused difficulties in standardization of scarlet fever toxin preparations.

2. Evidence presented in this paper and in works previously published points to repeated injections as a factor in an altered reactivity of the skin.

3. The following safeguards against difficulties in standardization tests in institutions where repeated injections are practiced are suggested: Two injections of each unknown preparation should be made against two injections of the standard toxin in each individual in a test. Therefore, only two toxin preparations could be standardized in a single test with a group of individuals, instead of the five preparations ordinarily tested.

4. Two readings of results—in 7 to 8 hours and in 21 to 22 hours—are preferable when convenient and practical. A more suitable interval between injections and single readings might be worked out through a greater number of observations.

We wish to acknowledge our indebtedness to Dr. Morris Siegel and Dr. I. J. Brightman for their very helpful cooperation in the matter of arranging for tests, and especially for the precision and care with which the injections were made.

REFERENCES

1. Joe, Alexander: The Dick Test, Lancet **2**: 1321, 1925.
2. Zoeller, C., and Manoussakis: Toxine streptococcique et immunité locale, Compt. rend. Soc. de biol. **93**: 24-25, 1925.
3. O'Brien, R. A.: The Titration of Scarlet Fever Toxin, J. Hyg. **29**: 357, 1930.
4. McGibbon, J. P.: The Anomalous Features of the Dick Reaction, J. Hyg. **34**: 30, 1934.
5. Smiley, H. E.: Specificity of the Dick Test, J. Lab. & Clin. Med. **20**: 589, 1935.
6. Ker, Frank L.: Some Observations of the Dick Test, Brit. M. J. **1**: 852, 1937.
7. Dick, G. F., and Dick, G. H.: Local Immunity to Scarlet Fever Toxin, J. Infect. Dis. **62**: 83, 1938.
8. Zingher, Abraham: The Dick Test in Normal Persons and in Acute and Convalescent Cases of Scarlet Fever, J. A. M. A. **83**: 432, 1924.
9. Moriwaki, J.: Reappearance of Previously Positive Dick's Test and Blanching at the Site of Previously Positive Test at Later Development of Scarlet Fever, Japan M. World **7**: 65, 1927.
10. Ferry, N. S.: Reappearance of Reaction at Site of Previous Dick Test Coincident With Appearance of Measles Rash in a Case of Measles, J. A. M. A. **87**: 241, 1926.
11. Toomey, John A.: Reappearance of a Positive Dick Test, J. A. M. A. **87**: 941, 1926.
12. Goodall, E. W.: An Abnormal Dick Reaction, Brit. J. Child. Dis. **33**: 207, 1936.

MODIFICATION OF HUDDLESON'S OPSONOCYTOPHAGIC REACTION*

Royall M. Calder, M.D., San Antonio, Texas

THE opsonocytophagic reaction, introduced in 1933 by Huddleson and his co-workers,[1] has assumed a position of considerable importance in the diagnosis of brucellosis and in gauging results of treatment. However, the technique as originally described is difficult, in that scrupulously clean glassware is essential, the examination of leucopenic bloods is time-consuming in thin preparations, and there is danger of infection if the two cover-slip method of spreading the blood is employed. These difficulties are largely eliminated by the following modification.

*From the Clayton Foundation for Research, Petroleum Bldg., Houston, Texas.
Received for publication, July 20, 1939.

Cleaning of Glassware.—The glass slides may be cleaned satisfactorily by washing well in soap and hot water. Sodium hexametaphosphate (sold by numerous scientific supply houses under the trade name of Calgon and Calgonite) is an aid if the water is hard. After drying, the slides are washed in automobile antifreeze solution (such as Du Pont's "Five Star"), which is an inexpensive excellent grease solvent, and polished with a clean linen cloth.

Preparation of Spreads.—After incubation of the mixture of blood and organisms as described by Huddleson, several large drops of cell sediment are pipetted from the bottom of the tube, placed on one end of a slide, and spread in the usual manner by dragging the blood with another slide held at an angle, stopping about a half inch from the end of the bottom slide. Then the top slide is held loosely and dragged back with an uneven motion, so that a very thick layer of blood results.

The slides are dried as quickly as possible under an electric fan. They are then immersed in a solution of 1 per cent acetic acid in distilled water contained in a Coplin staining jar and allowed to remain for several minutes until dissolution of the red blood cells is effected; the addition of 5 per cent commercial formalin to this solution contributes to safety but is probably unnecessary and has the disadvantage of causing additional shrinkage of the leucocytes. The slides are washed through at least three changes of distilled water to remove all traces of acid (excess of acid interferes with staining).

The preparations are stained with Bordet-Gengou's carboltoluidine blue, as suggested by Dr. Alice Evans. This stain is superior to Hasting's or Wright's stains because cytoplasmic structures, such as normal and "toxic" granules, do not take the stain and hence cannot be confused with engulfed bacteria. Bordet-Gengou's toluidine blue is prepared by dissolving 5 Gm. of toluidine blue in 100 c.c. of 95 per cent ethyl alcohol, 500 c.c. distilled water, and 500 c.c. 5 per cent phenol. It should be filtered after standing for one or two hours, and diluted with two parts of distilled water before being used for staining. The slides are stained for fifteen seconds, and washed with distilled water.

Preparations made in this manner are easily read and interpreted after experience, for the leucocytes and bacteria stand out as deeply stained structures against a faint, blue background. If care is taken to select isolated leucocytes for examination, avoiding clumps of cells and agglutinated organisms, the degree of phagocytosis can be determined speedily and accurately.

REFERENCE

1. Huddleson, I. F., Johnson, H. W., and Hamann, E. E.: Study of Opsono-cytophagic Power of Blood and Allergic Skin Reaction in Brucella Infection and Immunity in Man, Am. J. Pub. Health **23**: 917, 1933.

DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

SYPHILIS, Serologic Discrepancies in, Crawford, G. M., and Ray, L. F. J. A. M. A. **113**: 1715, 1939.

Of 2,862 new patients admitted to the syphilis clinic at the Massachusetts General Hospital in a two-year period, 335 late cases showed a positive Hinton and a negative Wassermann reaction. This group was studied exhaustively for additional evidence of syphilis. Nearly 50 per cent of these patients were found to exhibit a history or physical or laboratory evidence of the disease along with a repeatedly positive Hinton reaction. Another 25 per cent were diagnosed as syphilitic on the basis of a persistently positive Hinton reaction alone. In 12.5 per cent a vacillating serologic reaction and lack of other evidence created indecision as to the presence or absence of syphilis. In the remaining 14.3 per cent the one initial positive reaction, followed by repeated negative reactions, was held to be a technical error.

The average age of the entire group was 42.8 years. The known average duration of syphilis in the clinically active group was 21.5 years in acquired cases. The average age of congenital syphilitic patients with active disease was 25.3 years, the oldest of whom was 30.

False positive reports were computed to allow for the widest possible margin of error. All undecided cases and all technical errors were included. Comparing this number with all new specimens examined by the laboratory during the time of the study, a figure of 0.42 per cent of false positives was obtained. This corroborated clinically the high degree of specificity attained by the Hinton test in congresses on serologic tests.

STREPTOCOCCUS VIRIDANS, Bacteriemia Following Extraction of Teeth, Palmer, N. D., and Kemple, M. J. A. M. A. **113**: 1788, 1939.

Seventeen per cent of a group of 82 patients who had not more than two teeth extracted had transient bacteriemia. In 13.4 per cent of these the organism was *Streptococcus viridans*. In four cases of subacute bacterial endocarditis the onset of septic symptoms dates from the time of dental manipulation.

Bacteriemia is present in an appreciable percentage of cases of severe oral sepsis independent of operation. Organisms "leak" into the circulation from such foci. Traumatization of the diseased alveoli laden with *Streptococcus viridans* causes dispersion of these organisms through the blood stream in a high percentage of cases. The percentage is roughly parallel to the severity of the infection in the gums and to the extent of the operative procedure. This may be purely mechanical dispersion rather than invasion. In persons with a normal vascular system and a normal defense mechanism, this form of bacteriemia is relatively unimportant. The circulation is usually cleared of the invaders within a few minutes. Hypersusceptibility of the tissues due to long-continued oral sepsis is speculated upon by Cecil. In persons who have pre-existing rheumatic valvular lesions or congenital defects in the heart, localization of the organism on such vulnerable areas during the transient bacteriemia, which so often follows dental operations, may herald the beginning of an engrafted bacterial endocarditis.

MALARIA, Sulphanilamide (Prontosil) in the Treatment of, Niven, J. C. Inst. M. Research, Federated Malay States, Bull. 4, 1938.

An investigation is described in which 80 persons with acute malaria treated with prontosil are compared with 68 persons treated with quinine bihydrochloride.

It is found that prontosil is not so efficient as quinine in *P. falciparum* malaria.

Prontosil is still less effective in *P. vivax* and *P. malariae* malaria.

Prontosil is not an efficient gametocide in either *P. falciparum* or *P. vivax* malaria. Mosquitoes fed on "crescent" carriers after seven-day treatment with prontosil were found to be readily infected.

No toxic effects were noted.

It is concluded that, although prontosil has some lethal action on malaria parasites, especially *P. falciparum*, it has no place in the practical treatment of malaria owing to its low efficiency, possible toxicity, and relatively high cost.

GONORRHEA, Sulphanilamide and Its Derivatives in the Routine Treatment of, in the Tropics, Poynton, J. O. Inst. M. Research, Federated Malay States, Bull. 5, 1938.

An attempt is made to show that sulfanilamide therapy is a better and cheaper routine treatment for gonorrhea in the hospitals of Malaya than other forms of treatment at present employed.

In a series of 98 persons treated with sulfanilamide, 70 per cent were clinically cured, the majority within a fortnight.

A series of persons treated with uleron showed this drug to be far less effective than sulfanilamide.

No dangerous toxic effects were seen either with sulfanilamide or with uleron, and minor effects, such as giddiness or fever, disappeared immediately on suspending the treatment.

The results suggest that the Tamils tend to respond less favorably and are more liable to minor toxic effects than are the Chinese.

Total and differential leucocyte counts before and after treatment showed, in the majority of instances, that with sulfanilamide therapy these counts tend to return to normal as the result of a course of treatment.

In using sulfanilamide as a routine treatment for gonorrhea it is important that a definite schedule be followed and that certain precautions be observed. These are discussed.

In the course of completing these series of cases it became clear that sulfanilamide therapy is far more popular with Asiatic patients than previously employed forms of treatment. It is suggested that if it were introduced as a routine it would incite afflicted persons to present themselves at an earlier stage of the disease, reducing the incidence of serious complications, the activities of quack medicine vendors, and possibly, over a number of years, influencing the incidence of the disease by reducing the number of chronic infections at large.

It is suggested that sulfanilamide is unsuitable for self-administration and should not be available for open purchase.

Other sulfanilamide derivatives have been the subject of favorable reports, in particular M & B 693. At present the price of the latter militates against its routine employment in tropical hospitals for the treatment of gonorrhea.

This investigation was completed in the middle of 1938. Since that time many papers of importance on the subject have appeared, especially in connection with the value of M & B 693.

BONE MARROW: Limitations of Biopsy of Sternal Marrow, Kandel, E. V., and LeRoy, G. V. Arch. Int. Med. **64**: 121, 1939.

It is the authors' impression and belief that extremely thorough clinical investigation of patients robs biopsy of sternal marrow of much of its value. In their hands it has more commonly been a link, and often a superfluous link, in the diagnostic chain than it has been the sole means of establishing a diagnosis. The knowledge that potent products for the treatment of pernicious anemia convert the megaloblastic marrow to erythroblastic marrow within, usually, twenty-four hours, is valuable in clinical research. It permits more rapid assaying of new remedies and allows one to expose his patients to a minimum of risk during the testing period. With severely anemic patients there is an obvious advantage in not waiting seven or ten days for the expected response from experimental therapy.

The techniques for biopsy of marrow, the normal differential counts of material so obtained, and the limitations of the methods are discussed.

The authors' experience is reviewed, and 28 examples illustrating the uses, benefits, and disadvantages of biopsy of sternal marrow are presented.

RABIES, Improved Methods in the Diagnosis of, Willett, J. C., and Sulkin, S. E. J. Am. Vet. M. A. **95**: 659, 1939.

The white mouse is the animal of choice in the laboratory diagnosis of rabies, Swiss mice being of no practical advantage.

Demonstration of Negri bodies in the test animal (white mouse) is the only dependable criterion for a positive diagnosis, since clinical manifestations are frequently too variable and indefinite to serve any expedient means of diagnosis other than the guinea pig method.

A simple procedure is described for demonstrating rabies virus in grossly decomposed animal brains.

Ether in final concentration of 10 per cent exerts a definite bactericidal effect upon contaminated dog brains after exposure for two hours at 4° C. This concentration has no effect upon the virulence of the rabies virus.

A simple technique is described for removing animal brains.

The following method is recommended for treating contaminated brains before intracerebral injection into animals. The central portion of the hippocampus major (cornu ammonis) is dissected out, and a small piece removed for microscopic examination and for glycerination. The remaining portion of the horn is emulsified in 10 c.c. of nutrient broth and allowed to stand for one hour at room temperature. The supernatant liquid (after larger clumps have settled) is pipetted into a stoppered 15 c.c. (0.5 ounce) centrifuge tube, and 10 per cent ether (about 1 c.c.) is added. The tube is then shaken thoroughly and stored in the refrigerator for two hours. After centrifugation at low speed the supernatant liquid below the ether layer is removed for animal inoculation.

The following instruments are required: (1) A heavy board (2 inches by 12 inches by 16 inches) with two 6 inch nails, located 2 inches from one end and about 4 inches apart; (2) one scalpel (Bard-Parker No. 4 handle, No. 20 blade); (3) one pair of rubber gloves; (4) one tablespoon; and (5) one metal adjustable hack saw (8 inch blade).

The animal is placed on the operating table with its head between the nails on the paper-covered heavy board. The surface of the head is then thoroughly moistened with 4 per cent cresol. With a heavy scalpel a sagittal incision is made midway between the eyes and ears, cutting through the skin, fascia, and muscles of the cranium. The brain may be exposed by sawing sagittally through the parietal bones in the region of the frontoparietal suture. The nerves on the under side of the cerebrum, tentorium, and spinal cord can be severed by forcing the tablespoon along the floor of the cranial cavity. The brain is then lifted out and transferred to a receptacle which is provided for this purpose.

LEPTOSPIROSIS, Canine, in the United States, Meyer, K. F., Stewart-Anderson, B., and Eddie, B. J. Am. Vet. M. A. **95**: 710, 1939.

The following procedures are recommended:

Silver Impregnation Method for the Demonstration of Leptospira:

1. Fix blocks of tissues, not exceeding 1 cm. in diameter, in 10 per cent formalin in distilled water for several days or weeks, preferably three months.

2. Harden in absolute alcohol for twenty-four to forty-eight hours.

3. Pass through alcohol (70 per cent, 50 per cent, 30 per cent) to distilled water until the tissues are freed from alcohol.

4. Immerse in a 0.25 to 0.5 per cent silver nitrate solution in distilled water for eighteen to twenty-four hours at 37° C. in the dark.

5. Wash thoroughly in distilled water, changing the water several times.

6. Place in 0.5 to 1 per cent hydroquinone in 50 per cent alcohol for eighteen to twenty-four hours at room temperature.

7. Wash in 70 per cent alcohol; dehydrate in 95 per cent and absolute alcohol; clear in xylol; embed in paraffin.

8. Prepare very thin sections (3 to 4 microns). Remove paraffin and mount in balsam.

Schüffner Modification of Verwoort's Medium:

Use 1.5 liters tap water (free from chlorine; rain water may be used instead of distilled water) and 1.5 Gm. Witte peptone. Boil.

Add 6 c.c. phosphate mixture composed of 0.35 Gm. potassium phosphate (monobasic), 1.33 Gm. sodium phosphate (dibasic), and 100 c.c. distilled water.

Boil. Add 300 c.c. Ringer's solution composed of 0.8 Gm. sodium chloride, 0.02 Gm. calcium chloride, 0.02 Gm. potassium chloride, 0.02 Gm. sodium bicarbonate, and 100 c.c. distilled water.

Continue boiling. Add 150 c.c. Sörensen's buffer solution, pH 7.2. Boil until precipitation is complete (about thirty minutes). Cool in icebox overnight. Filter. Test pH, which should be 6.8 to 7.2. Bottle. Autoclave at 15 pounds for fifteen minutes. The medium may be kept in this state in capped bottles until needed. For use add 8 to 10 per cent sterile rabbit serum, tube in 2.5 to 3 c.c. amounts, and inactivate for thirty minutes in a 56° C. water bath.

Test for sterility. Transplant, using a 1 c.c. pipette.

Leptospira Agglutination Test—Porcelain-Plate Method:

Antigen: Use a 4- to 6-day-old culture of each strain. Use both live and formalin-killed antigen (3 drops commercial formalin per 10 c.c. culture). Incubate plates containing live antigen for two hours at 37° C., and examine each dilution by dark field for lysis. Incubate plates containing formalin-killed antigen for four hours at 37° C. Examine each dilution by dark field for agglutination or clumping.

Test: For each serum specimen place four test tubes in a rack. Use Verwoort-Schüffner buffer solution without serum as diluent.

Tube	1	2	3	4
Buffer	1.2 c.c.	0.9 c.c.	0.9 c.c.	0.9 c.c.
Serum*	0.3 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.
Dilution	1:5	1:50	1:500	1:5,000

Use two porcelain plates for each specimen, one for live antigen, one for formalinized killed antigen.

CL. TETANI, Serological Identification of, Maclennan, J. D. Brit. J. Exper. Path. 20: 26, 1939.

The H and O agglutinin reactions of *Cl. tetani* have been examined, and the findings of Gunnison (1937) confirmed.

The O antigens of *Cl. tetani* are apparently species-specific, and have not been found in any of nine other species of terminally-spored anaerobes.

By means of suitable O antisera a new serologic type of *Cl. tetani*—type X—has been identified.

TYPHOID BACILLI, A Leucocidal Toxin Extracted From, Dennis, E. W., and Senekjian, H. Am. J. Hyg. 30: 103, 1939.

A potent leucocidal factor has been demonstrated in trichloracetic acid extracts of typhoid bacilli.

Leucocidal extracts of bacilli are analogous to leucocidal culture filtrates in selective lethal effect on the pseudo-eosinophilic granulocytes, and in thermostability.

Purification of the trichloracetic acid extract results in increased potency of the toxic somatic antigen-complex, but in loss of leucocidal activity. The crude extract represents a mixture of these two factors.

Typhoid leucocidin is a component of the typhoid bacillus, and its presence in culture filtrate is probably the result of early autolytic processes.

The leucocidal substance in trichloracetic acid extracts is not in an antigenic state.

Leucocidin can be demonstrated in similar extracts of *Salmonella typhimurium, Sal. schottmülleri, Sal. suipestifer* (voldagsen strain), *Sal. enteritidis, Sal. gallinarum,* and *Sal. paratyphi,* listed in order of decreasing leucocidal activity.

*Transfer 0.1 c.c. from tube 2 to 3 and the same amount from 3 to 4.

TUBERCULOSIS, Sulfanilamide in Clinical, Nayer, H. R., and Steinbach, M. M. Am. Rev. Tuberc. **40**: 470, 1939.

Eight patients with extensive bilateral pulmonary tuberculosis were treated with sulfanilamide for a period of from ten to seventy-one days.

As far as the authors could evaluate the results, there was no apparent effect, either beneficial or harmful, upon the disease.

STREPTOCOCCI, Hemolytic, Studies on, From Human Sources, Rudd, G. V., White, C., and Ward, H. K. Australian J. Exper. Biol. & M. Sc. **17**: 25, 1939.

Seven hundred and eighty-four strains of group A hemolytic streptococci isolated in Sydney, Australia, were examined for their serologic type. Five hundred and eighty-nine belonged to one of Griffith's types, 92 belonged to types not represented in Griffith's classifications, and 103 could not be typed.

Griffith's types 2, 3, 13, 14, 15, 18, 19, 24, "Quinn," and "Coggins" have not been isolated in Sydney up to the present.

Types 17, 11, 1, and 4 accounted for 75 per cent of the scarlet fever strains, while these types accounted for only 12 per cent of strains from other sources.

Evidence is submitted suggesting that capsulated organisms are likely to be more infectious than noncapsulated organisms.

The technical problems of typing are discussed.

BLOOD, Biochemical Changes Occurring During the Storage of Human, Bick, M. Australian J. Exper. Biol. & M. Sc. **17**: 321, 1939.

The storage of human blood is accompanied by a decrease in the glucose content of the blood, an increase in the reduced glutathione, uric acid, creatinine, nonprotein nitrogen, and inorganic phosphate. There is little increase in the urea content of the blood.

The onset of hemolysis may be delayed by the addition of glucose. The optimum amount of glucose is 0.6 to 0.7 per cent. A small part only of the increase in nonprotein nitrogen is accounted for by the increase in urea, creatinine, and uric acid. Hemolysis of the erythrocytes generally occurs when the nonprotein nitrogen reaches a concentration of 55 to 60 mg. per 100 c.c. blood.

The biochemical changes that take place may be attributed to the erythrocytes, but the essential nature of the process is unknown.

As the inhibition of hemolysis does not seem possible as yet, it should be stressed that the rigorous cleansing of all glass and purification of reagents must be undertaken to procure consistent and reproducible results in this work.

HEMOPHILIA, A Study of the Clotting Defect in: The Delayed Formation of Thrombin, Brinkhous, K. M. Am. J. M. Sc. **198**: 509, 1939.

A quantitative study of changes in the prothrombin titer of hemophilic blood shows that its prothrombin is converted very slowly into thrombin. This delayed prothrombin conversion can be corrected by adding less than 1 mg. of crude thromboplastin to 100 c.c. of hemophilic blood. Evidence indicates that the prothrombin and fibrinogen are normal in amount and in reactivity in this disease. There is no excess antithrombin. Emphasis is placed upon the formed elements of the blood and the sluggishness with which they liberate thromboplastin.

REVIEWS

Books and Monographs for Review should be sent direct to the Editor,
Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

A Textbook of Pathology for Nurses*

THIS is a well-written and well-organized little book, the purpose of which is to present to the student nurse in simple language, a working concept of the mechanism of disease.

Based upon the author's course in the Mt. Sinai Hospital of New York, it fulfils its purpose satisfactorily and should meet a favorable reception from instructors in training schools for nurses.

Pathology†

THE subtitle of this book, "An Introduction to Medicine and Surgery," indicates the manner in which the authors have approached their subject. For, as is now rather generally appreciated, pathology is no longer regarded as synonymous with morbid anatomy, but appreciated as an essential phase in the study of disease in the living patient.

In the organization of the book the authors have accepted a compromise between the conventional separation into general and special pathology and that treating as one all conditions due to the same cause. The result is an effective text, not only for the student to whom it is primarily addressed, but also for the physician at large.

It is recommended as a good book.

Proctoscopic Examination‡

BECAUSE of the generous margins and large type, this small book is even smaller than it appears to be. While there is much that is good in the text, the book gives one the impression of being an expansion of a synopsis for lectures and, as a whole, it is a somewhat fragmentary and uneven exposition of the subject. The chapters on proctoscopic examinations are the best portion of the text. There will be some reluctance to accept neoplasms as *common* causes of diarrhea, and in comparison to the space given to other conditions of importance (for example, amoebic dysentery and amoebic colitis) that given to the discussion of malignancy seems disproportionate though not in itself exhaustive.

This book could well be taken as a synopsis from which a better and more comprehensive work could be written.

As it stands, one must reluctantly admit that it is not among the outstanding contributions in this field.

---

*A Textbook of Pathology for Nurses. By Coleman B. Rabin, B.S., M.D., Lecturer in Pathology, Mt. Sinai Hospital School of Nursing; Associate in Medicine and Assistant Radiologist to the Mt. Sinai Hospital; Associate Radiologist to Harlem Hospital and Associate in Medicine, Columbia University, New York. Cloth, ed. 2, 260 pages, 68 illustrations, $1.75. W. B. Saunders Co., Philadelphia, Pa.

†Pathology. An Introduction to Medicine and Surgery. By J. Henry Dible, M.B., F.R.C.P. Professor of Pathology, University of London; and Thomas B. Davie, M.D., M.R.C.P., Professor of Pathology, University of Liverpool. Cloth, 931 pages, 374 illustrations, including 8 color plates, $10.00. P. Blakiston's Son & Co., Philadelphia, Pa.

‡Proctoscopic Examination and Diagnosis and Treatment of Diarrheas. By M. H. Streicher, M.S., M.D., Assistant Professor of Medicine, University of Illinois, College of Medicine and Research and Educational Hospital, and Department of Surgery, Grant Hospital of Chicago. Cloth, 150 pages, 39 illustrations, $3.00. Charles C. Thomas, Springfield, Ill.

Medicolegal and Industrial Toxicology*

THIS is a compact and well-planned discussion based upon practical experience. While not encyclopedic in scope, it is in many respects a *multum in parvo*, as indicated by the chapter headings: Poisons and Drugs, Criminal Investigations, Medicolegal Examinations of Miscellaneous Nature, Industrial Poisoning, and Occupational Diseases.

As a ready reference text, to be supplemented by larger and more comprehensive volumes, this book should be of interest and value to industrial physicians, coroners, pathologists, lawyers, insurance adjusters, and all who are concerned with the fields covered. A good book to have at hand.

Blood Groups and Blood Transfusions†

THIS book needs no introduction, for it has been, since the appearance of its first edition, a standard and authoritative text on the subject.

Extensively revised, the text is one which may well be said to be invaluable to the physician, clinical pathologist, medicolegal expert, and laboratory worker, and may justly find a place on the reference shelf of the legal library.

The section on blood transfusions has been rewritten to include the latest developments and constitutes a complete survey of the subject.

Blood grouping is thoroughly discussed with emphasis on its medicolegal implications as is also the grouping of blood stains. Research workers will find the discussion of blood groups in animals interesting and valuable.

All in all it is an excellent book and a "must" item.

The Neurogenic Bladder‡

THE purpose of this book is to give the reader a working knowledge of the value of cystometry in the differential diagnosis between neurogenic and non-neurogenic disease of the bladder.

It is a well-written monograph of practical importance and value, and can be recommended as an authoritative study.

Laboratory Diagnosis§

IT IS not surprising that this book, by reason of its practical approach to the problems of the student and to the problems of the physician as well, should have reached its third edition.

Increased in size and thoroughly and comprehensively revised, it retains the plan of its predecessors. The first section (322 pages) describes the clinical applications of laboratory procedures and their clinical interpretation. The remainder of the book is devoted to a description of technique.

---

*Medicolegal and Industrial Toxicology, Criminal Investigation, Occupational Diseases. By Henry J. Eilmann, Ph.D., Director, Physician's Laboratory Service of Toledo, Ohio; Lecturer in Bacteriology and Histology, Mary Manse College of Toledo. 324 pages, $3.00. P. Blakiston's Son & Co., Philadelphia, Pa.

†Blood Groups and Blood Transfusions. By Alexander S. Wiener, A.B., M.D., Serologist and Bacteriologist in the Office of the Chief Medical Examiner of New York City. Cloth, ed. 2, 306 pages, 52 figures. Charles C. Thomas, Springfield, Ill.

‡The Neurogenic Bladder. By Frederic C. McLellan, M.S., M.D., Instructor in Surgery, University of Michigan Medical School, Ann Arbor, Michigan. Cloth, 206 pages, 1 plate, 8 figures and numerous charts. Charles C. Thomas, Springfield, Ill.

§Laboratory Diagnosis. By Edwin E. Osgood, M.A., M.D., Associate Professor of Medicine and Head of the Division of Experimental Medicine, University of Oregon Medical School. Washable fabric, ed. 3, 676 pages, 10 colored plates, 27 text figures, $6.00. P. Blakiston's Son & Co., Philadelphia, Pa.

The book is so well known that it needs no extended description, but it can be said that this edition is as well organized, well written, and authoritative as its predecessors; it will undoubtedly maintain and enhance the popularity already acquired. A good book for the student, an excellent book for the clinician, and a valuable addition to the laboratory reference shelves.

What's Your Allergy?*

THIS is a book for the intelligent layman. While the title suggests a possible humorous approach, the book is a serious presentation in reasonably simple language, primarily for the enlightenment of the allergic patient. Statements made in the book are authentic, not exaggerated.

The chapter dealing with the mechanism and theory of anaphylaxis is obviously the most difficult portion of a semipopular book for presentation in simple and at the same time adequately descriptive phraseology. The author's solution to this problem of a clear-cut explanation of a subject, which physicians and even research students do not clearly understand, is not too involved and should leave the reader with the feeling that he has a fair idea of what may be happening in an anaphylactic reaction.

The reviewer fears that the authors may be placing too much hope in the therapeutic value of histamine, but the logic of their attitude is reasonable.

The book will make interesting reading for allergists, physicians in general, and nonmedical readers.

---

*What's Your Allergy? By Lawrence Farmer, M.D., Chief of Allergy Clinic, Lenox Hill Hospital, New York; and George J. Hexter. Cloth, 234 pages, $2.00. Random House, New York, 1939.